THE ROLE OF COORDINATED REGULATION AND AROMATIC METABOLITES IN ACTIVATING THE *MAR/SOX/ROB* REGULON OF *ESCHERICHIA COLI*

BY

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DISSERTATION

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Abstract

Bacterial resistance to antibiotics has focused primarily on the role of acquired genetic elements contained in transposons and plasmids. In contrast to this view, many bacterial species are equipped with intrinsic mechanisms to survive exposure to a wide variety of antimicrobial compounds. This form of resistance is mediated through regulated expression of efflux pump systems, reducing enzymes, and enzymes in cellular metabolism. Modulating this response in *Escherichia coli* are three homologous, transcription factors: MarA, SoxS, and Rob. Together, these transcription factors serve as master regulators of the extensive *mar/sox/rob* regulon that has been directly implicated in multidrug resistance found in clinical and laboratory isolates. In this work, we examine the degree of genetic cross-talk between these regulatory systems and the cooperative role of these three transcriptional regulators in activating downstream targets.

The overarching goal of this work is to provide an integrated model for the *mar/sox/rob* regulatory network. First, the role of MarA, SoxS, and Rob in cross-regulating and auto-regulating expression from the *marRAB*, *soxRS*, and *rob* loci is explored. Previous evidence has suggested the potential for a fully interconnected transcriptional regulatory network between *marRAB*, *soxRS*, and *rob*. Using a genetic approach, the transcription-level interaction between the

marRAB, *soxRS*, and *rob* systems was dissected and a more complete model is proposed. As a corollary, evidence is presented to support a model where MarA serves a conditional auto-repressor of its own expression. Similarly, genetic and biochemical evidence is presented showing the global nutritional regulator, cyclic AMP receptor protein (CRP) interacts directly with the *marRAB* promoter region. Second, the role of MarA and Rob in coordinately regulating the reduction of OmpF expression during drug exposure is examined. The canonical model for this event argues that up-regulated expression of MicF (a small RNA regulator of OmpF translation), mediated by MarA, SoxS, and Rob, is the causal agent of OmpF reduction. Evidence is here provided that MarA and Rob function as independent pathways for *micF* promoter activation. Likewise, data is presented to suggest the possibility of a MicF-independent pathway for OmpF reduction that is regulated by MarA. Additionally, the reduction in OmpF expression in *tolC* mutants is found to be the result of Rob-dependent activation of MicF. Finally, genetic and biochemical data is presented that demonstrates the role of aromatic metabolites in activating the *mar/sox/rob* regulon through direct interaction with the repressor protein, MarR. Collectively, these results provide continuing steps towards an integrated view of the *mar/sox/rob* regulon and cellular physiology.

For everyone who fought with me.

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Chapter 1: An introduction to the *mar/sox/rob* regulon

1.1 General perspectives on antibiotic resistance

Bacterial resistance to antibiotics has been a persistent problem in clinical and public health situations for decades. Since the implementation of penicillin in the 1940's to treat streptococcal septicemia (Florey, 1941), countless resistant strains of both commensal and pathogenic bacteria have emerged for nearly all antibiotics currently used in medical practice (Levy and Marshall, 2004, Livermore, 2009, Livermore, 2004). As treatment regiments become increasing limited, the need to comprehend the mechanisms of resistance and possible alternative treatment strategies has become paramount.

Bacterial resistance to antibiotics has been observed to occur through two primary modes; acquired resistance and intrinsic resistance (Alekshun and Levy, 2007, Nikaido, 2009). Acquired resistance is the result of beneficial chromosomal mutations or more commonly the acquisition of genes encoding antibiotic resistance determinants. These determinants are typically encoded on mobile genetic elements such as plasmids, bacteriophage and transposons (Levy and Marshall, 2004, Alekshun and Levy, 2007, Nikaido, 2009). Intrinsic resistance, on the other hand, is a process where bacteria can initiate changes in cellular metabolism and physiology to survive exposure to antimicrobial compounds (Alekshun and Levy, 2007, Nikaido, 2009). The result is often no single genetic

determinant can account for resistance to certain classes of antibiotics. A defining feature of intrinsic resistance is that the genes required for this phenotype are naturally occurring in the host's genome.

Due to the complexity of intrinsic resistance, only a limited number of associated mechanisms have been extensively studied. Active mechanisms such as drug resistance efflux pumps being the most fully understood (Paulsen, 2003). Recently, efforts have been made to shed light on other physiological changes occurring within cells during adaption to multi-drug environments (Kohanski et al., 2010, Kohanski et al., 2007, Kohanski et al., 2008, Bollenbach et al., 2009, Liu et al., 2010, Tamae et al., 2008). Combining classical microbiology with systems-level measurements has allowed an ever more complete picture the changes occurring inside bacterial cells during drug exposure.

Though researchers have on one hand observed many of the physiological changes that can occur on antibiotic exposure, mapping these changes to changes in gene regulation has remained an uncompleted task (Dwyer et al., 2008, MacLean et al., 2010). The overarching goal of this work is to form a more complete picture of how these changes may be regulated at the genetic level. In the case of *Escherichia coli*, three independent genetic regulatory systems have been implicated in causing natural, low-level resistance to a broad spectrum of antibiotics. These systems are the *marRAB*, *soxRS*, and *rob* regulatory networks. Encoded in these systems are genes for three,

homologous transcriptional regulators MarA, SoxS and Rob. Each system is activated in response to different chemical stimuli, yet these three transcription factors regulate a vast, overlapping network of genes required to survive in toxic environments. A documented, but poorly understood, feature of these networks is their capacity to regulate the expression of one another. This work aims to explore this genetic interconnectivity and use this as a basis to understand how the network manages a large downstream regulon. Further, this dissertation attempts to expand our understanding of the possible role of intermediary metabolism in activating the *marRAB* genetic circuit. Collectively, this work attempts to define a clear genetic regulatory picture of the intrinsic multi-antibiotic resistance system of *E. coli*.

1.2 The two general mechanisms of antibiotic resistance

Acquired antibiotic resistance. Acquired antibiotic resistance is the result of beneficial mutations to genes encoding targets of antibiotic action or more commonly bacterial cells receiving heterologous DNA containing genes encoding resistance determinants from the environment (Alekshun and Levy, 2007, Levy and Marshall, 2004). These resistance determinants, or genes, are typically located in mobile genetic elements such as plasmids and transposons which can be introduced to bacterial cells by bacteriophage infection, conjugal transfer from

neighboring bacteria, or natural uptake of DNA from the environment (Alekshun and Levy, 2007, Levy and Marshall, 2004).

Acquired resistance mechanisms can be generally considered as heritable forms of resistance (**Figure 1.1**). As opposed to their inducible, or intrinsic, counterparts, a fundamental component of the acquired resistance mechanism is that permanent changes are made to the host DNA, resulting in the production of equally resistant progeny. Partly because of these permanent genetic changes, acquired resistance mechanisms have readily traceable sources and have remained the most fully understood.

The first strides towards understanding the mechanisms of acquired resistance were made in Japan during the late 1950's. During this time, an increase in cases of septic dysentery that were not treatable with sulfonamide antibiotics had been observed. To determine the cause of emerging antibiotic resistant strains of *Shigella*, researchers examined numerous isolates and discovered that the resistance determinants were disseminated through conjugal transfer of plasmids (Watanabe, 1963). From this point on, other mobile elements such as transposons and integrons have been found in numerous bacterial species both in clinical settings and the environment (Alekshun and Levy, 2007, Levy and Marshall, 2004, Mazel, 2006). The rapid transfer of these genes throughout diverse bacterial species, and the capacity of these elements to incorporate ever-increasing numbers of antibiotic resistance genes, has resulted

in large-scale efforts to discover novel treatment strategies (Alekshun, 2005, Alekshun and Levy, 2007, Levy and Marshall, 2004). Although these problems have significant clinical merit, they are beyond the scope of this work and will not be covered further.

Intrinsic antibiotic resistance. Intrinsic antibiotic resistance is a mechanism fundamentally different from acquired resistance. The primary source of divergence between these mechanisms is that intrinsic resistance is non-heritable and typically inducible. By non-heritable resistance, it is meant that the resulting progeny of drug-exposed bacterial cells are genetically identical and equally as sensitive to antimicrobials as the unexposed parents. Likewise, the term inducible is meant to imply that level of resistance to particular antimicrobial compounds is strongly dependent on the chemical environment of cell. For example, and relevant to this study, the presence of salicylates in bacterial growth medium is known to induce resistance to a wide variety of drugs. The progeny of these salicylate exposed cells, however, remain sensitive to the same drugs in the absence of salicylates.

An important outcome of the difference between the acquired and intrinsic mechanisms is pin-pointing specific determinants of intrinsic resistance is difficult. The primary reason for this is that intrinsic resistance typically results

from the concerted action of numerous cellular systems. Acquired resistance, on the other hand, is often due to singular, inherited factors.

Two prototypical examples will be covered here to illustrate the nature of intrinsic resistance; formation of persister populations and regulated multidrug resistance. Early studies on the use of antibiotics in clinical practice found subgroups of bacterial cells in infected patients that survived antibiotic exposure, yet remained sensitive to drug treatment – known as persister populations (Bigger, 1944). Given recent advances in genomic and systems-level methodologies, the elucidation of a genetic basis for persister cell development has advanced significantly. Based on current findings, it is clear that a number of host factors are at play in producing the formation of persister cells in bacterial populations including metabolic enzymes and chromosomally encoded toxin-antitoxin systems (Lewis, 2008, Lewis, 2010). For example, Spoering and colleagues have demonstrated that enzymes involved in central metabolism such as glycerol-3-phosphate dehydrogenase (GlpD) are involved in the formation of persister cells (Spoering et al., 2006). Presumably, intermediates in central metabolism may serve as signals for this developmental change. Likewise, the recent work of Dorr and coworkers has demonstrated the role of a toxin-antitoxin system encoded in the *E. coli* chromosome as a determinant of ciproflaxin-induced persister cell formation (Dorr et al., 2010). In both cases, it is believed that fluctuations in the expression of these genes is responsible for the formation of dormant subpopulations of bacterial cells. Despite the discovery of specific

mechanisms, large-scale understanding of persister development remains unclear (Lewis, 2010).

A second example, and the focus of the remaining discussion, is regulated multidrug resistance. Regulated resistance mechanisms are ubiquitous in bacterial species (Alekshun and Levy, 2007, Nikaido, 2009, Paulsen, 2003). The fundamental basis for regulated resistance is that cells sense the presence of an intracellular or extracellular concentration of toxic chemical(s) and initiate changes in gene expression to alter aspects of cellular physiology and metabolism to combat the action of the toxin(s). For *E. coli*, and closely related bacterial species, this is mediated primary through the sensory action of transcriptional regulators and two-component regulatory systems, that in turn alter the gene expression of membrane bound efflux pumps and porins, as well as numerous metabolic and detoxifying enzymes (Nikaido, 2009, Miller and Sulavik, 1996, Hirakawa et al., 2003, Baranova and Nikaido, 2002, Nagakubo et al., 2002, Eguchi et al., 2003, Alekshun and Levy, 2007, Viveiros et al., 2007, Alekshun and Levy, 1999b). A result of these changes is that cells can be exposed to ever increasing concentrations of toxins such as antibiotics yet remain viable. The most extensively studied set of intrinsic resistance regulatory systems are the *marRAB*, *soxRS*, and *rob* systems of *E. coli* (Demple, 1996, Alekshun and Levy, 1999b, Randall and Woodward, 2002). Additionally, cryptic regulators, such as YdeO, have been repeatedly implicated in global screens for antibiotic resistance (Nishino et al., 2009, Masuda and Church, 2003, Masuda

and Church, 2002). Additionally, a recent global expression screen has shown that a number of two-component regulatory systems such as EvgS/EvgA, CpxA/CpxR and EnvZ/OmpR are involved in conferring multiple antibiotic resistance through activating a number of drug efflux pump systems (Hirakawa et al., 2003).

Persister cell formation and regulated multidrug resistance exemplify the primary characteristic of intrinsic resistance. In each case, a phenotype is induced by changes in gene expression. These changes are ultimately reversible as they do not result in permanent modification of the host's DNA, allowing the bacterial cells to remain sensitive to re-exposure to toxins and antimicrobials.

1.3 Observed mechanisms of intrinsic resistance in *Escherichia coli*

Multidrug efflux pumps.

The one universal mechanisms of intrinsic resistance in bacteria is the expression of multidrug efflux pumps (Poole, 2005, Nikaido, 2009, Paulsen, 2003, Piddock, 2006). Nearly all known bacterial species encode multidrug efflux pumps and their widespread abundance has allowed for their systematic classification (Paulsen, 2003). Although the role of efflux pumps in antibiotic-resistant clinical infection is clear, based on their observed expression in drug resistant isolates, the role they play in general bacterial physiology remains uncertain (Piddock, 2006).

Genomic and structural analysis has allowed for the classification of five families of bacterial efflux pumps. A large number of efflux systems are members of the ATP-binding cassette (ABC) family and utilize adenosine triphosphate (ATP) as an energy source to drive transport of chemicals across the membrane. Numerous pumps are members of the major facilitator superfamily (MFS) that are typically proton/chemical anti-porters that utilize chemiosmotic energy. The remaining families of resistance nodulation (RND), small multidrug resistance (SMR), and multidrug and toxic compounds effux (MATE) families are proton and ion-driven efflux systems.

In the context of this work, the AcrAB-TolC and EmrAB-TolC efflux systems of *E. coli* will be discussed. AcrAB-TolC and EmrAB-TolC are members of the RND and MFS families, respectively. Each pump system is TolC-dependent, in that they required the outer membrane pore, TolC, to transport chemical species across outer membrane of *E. coli* cells (Touze et al., 2004, Tanabe et al., 2009, Fralick, 1996). Both pumps are comprised of two subunits. AcrB and EmrB are inner membrane bound efflux pumps that contact TolC, whereas AcrA and EmrA are structural proteins for ancoring to the complex to the inner membrane (Touze et al., 2004, Husain et al., 2004). Although functionally similar, they differ in classification based on the homology of the inner-membrane pumps AcrB and EmrB to differing families of transporters.

Of the two systems, AcrAB-TolC has been the most extensively linked to multidrug resistance in *E. coli* and close relatives (Touze et al., 2004, Husain et al., 2004, Symmons et al., 2009). This is due in part to the wide substrate range of the AcrAB-TolC pump for such disparate compounds as solvents, dyes and detergents as well as numerous lipophilic antibiotics (Ma et al., 1995, Ma et al., 1996). Additionally, the expression of the *acrAB* operon, as well as the *tolC* gene, is regulated by numerous systems including *marRAB*, *soxRS*, and *rob* (Zhang et al., 2008, White et al., 1997). The net result is that AcrAB-TolC is capable to being expressed under numerous stressful chemical conditions and possesses the capacity to expel a wide range of toxins from the intracellular environment. This allows for *E. coli* to remain viable in many multidrug environments.

Alteration of outermembrane porin composition. The outer membrane of Gram-negative bacteria is abundantly studded with molecular pores known as porins. These porins allow for the passive diffusion of molecules from the extracellular environment into the host periplasm. In *E. coli*, two of these major porins OmpC and OmpF have been implicated in the intrinsic resistance phenotype (Cohen et al., 1988, Pages et al., 2008). Although structurally similar, OmpC and OmpF are known to facilitate differential rates of diffusion based on size with OmpC being the small molecular pore (<500 Da) and OmpF being the larger (<600 Da), respectively (Cowan et al., 1992). It has been shown that

selective expression of these porins allows *E. coli* cells to preferentially block the uptake of certain antibiotic compounds into the periplasm of Gram-negative bacteria (Pages et al., 2008).

The control of OmpC and OmpF expression has been an extensively studied phenomenon at both the genetic and biochemical levels (De la Cruz and Calva, 2010). The canonical model of OmpC and OmpF expression involves regulation of *ompC* and *ompF* transcription by the EnvZ/OmpR two-component regulatory system, responsive to extracellular osmolarity (Slauch et al., 1988, Slauch and Silhavy, 1989). EnvZ is the inner membrane-bound sensor-kinase which selectively phosphorylates OmpR (a DNA-binding transcription factor) in response to changes in the osmolarity of the surrounding medium (Forst et al., 1989). The phosphorylated form of OmpR (OmpR-P_i or phospho-OmpR) is capable of binding to the P_{ompF} promoter with high affinity and activating transcription of *ompF*. As the level of OmpR-P_i increases with osmolarity, OmpR-P_i is able to bind to low affinity sites in the P_{ompC} promoter resulting in upregulated transcription of *ompC* (Pratt and Silhavy, 1995). Likewise, OmpR-P_i binds to low affinity sites in P_{ompF} resulting in down-regulation of *ompF* transcription. The outcome is that under conditions of low osmolarity, cells preferentially express OmpF. And under conditions of high osmolarity, they express OmpC. Therefore this mechanism allows cells to exquisitely control the uptake of solutes from the external environment depending on the ratio of OmpC and OmpF expressed (Pratt et al., 1996).

Additional regulatory factors aid in controlling the OmpC/F ratio. Of particular note is the small regulatory RNA (sRNA), MicF (Pratt et al., 1996, Vogel and Papenfort, 2006). The MicF sRNA is known to bind to the 5'-untranslated region (5'-UTR) of the *ompF* mRNA thereby inhibiting translation of OmpF and effectively downregulating OmpF expression (Mizuno et al., 1984). MicF is expressed as the RNA poduct of the *micF* gene, divergently transcribed from the *ompC* gene (Mizuno et al., 1984). Due to their proximity to one another, *micF* and *ompC* share a common promoter control region, and consequently, both genes are regulated by OmpR-P_i (Matsuyama and Mizushima, 1985, Misra and Reeves, 1987). Additionally, the P_{micF} promoter is regulated by MarA, SoxS and Rob, products of the *marRAB*, *soxRS*, and *rob* systems (Cohen et al., 1993b, Martin and Rosner, 2002, Bennik et al., 2000, Li and Demple, 1994). The presence of additional transcriptional regulators of *micF* allow for OmpF expression to be regulated independently of the canonical EnvZ/OmpR mechanism.

Numerous early observations noted changes in outer membrane porin composition on antibiotic exposure. Particularly, Cohen and coworkers noted that certain antibiotic resistant mutants (constitutive mutants in *marRAB*) resulted in down-regulated expression of OmpF (Cohen et al., 1988). A similar phenotype was observed during exposure of *E. coli* cells to salicylate (a toxic, aromatic acid) (Rosner et al., 1991). Both of these phenotypes were later found to be mediated by the repressive action of MicF (Cohen et al., 1993b, Rosner et al., 1991). Later

efforts have shown that all three of the *marRAB*, *soxRS*, and *rob* systems are capable of regulating *micF* transcription (Martin and Rosner, 2002). However, the relative magnitude of the contributions MarA, SoxS, and Rob have towards P_{micF} activation under antibiotic exposure remains unclear.

Changes in cellular metabolism and expression of detoxifying enzymes. A strategy commonly employed during adaptation to toxic environments is the alteration of cellular metabolism and expression of detoxifying enzymes (Martin and Rosner, 2002, Viveiros et al., 2007, Kohanski et al., 2007, Pomposiello et al., 2001, Greenberg et al., 1990). In the case of metabolic changes, very little is known about the exact changes that occur. However, systems-level transcriptional analysis does indicate that changes in gene expression of many metabolic enzymes changes during antibiotic exposure (Pomposiello et al., 2001, Martin and Rosner, 2002, Kohanski et al., 2007). Conversely, the action of detoxifying enzymes has been widely explored *in vivo* and *in vitro*. Collectively, these two changes in cellular enzymatic activity likely work in concert to alleviate the stress placed on cells burdened by excessive chemical efflux and perturbation of membrane integrity.

In the context of this study, an important metabolic change concerns the alteration of carbon flux in central metabolism. Based on microarray and gene expression analysis, it is well documented the expression of enzymes such as

glucose-6-phosphate dehydrogenase (the *zwf* gene product) are upregulated during exposure to toxic compounds such as salicylate and redox cycling antibiotics (Pomposiello et al., 2001). Glucose-6-phosphate dehydrogenase is an enzyme that converts glucose-6-phosphate to D-glucono-δ-lactone-6-phosphate, an entry-level intermediate into the Entner-Doudoroff (ED) and Pentose-Phosphate (PP) pathways. Both the ED and PP pathways are known to generate more reducing equivalents of NAD(P)H during the oxidation of glucose than the Embden-Meyerhof-Parnas pathway. Based on the recent observations of Kohanski and coworkers who demonstrated a decrease in NADH levels of antibiotic exposed *E. coli* cells, an intriguing hypothesis is that upregulation of reducing pathways may alleviate stress imposed by changes in intracellular redox state (Kohanski et al., 2007). In a similar manner, two enzymes of the tricarboxylic acid (TCA) cycle, fumarase (*fumC*) and aconitase (*acnB*), are upregulated under similar conditions and believed to contribute to the same increase in cellular reducing power (Pomposiello et al., 2001, Martin and Rosner, 2002).

Similarly, during exposure to similar families of antimicrobial compounds, it is known that detoxifying enzymes such as superoxide dismutase are expressed (Greenberg et al., 1990). Superoxide dismutases (SOD) catalyze the conversion of superoxide anions to hydrogen peroxide, thereby indirectly reducing the production of hydroxyl radicals and subsequent DNA damage. For the perspective of this study, the Mn^{2+} containing SOD (*sodA*) is upregulated during

treatment of cells with compounds such as salicylate and redox cycling antibiotics (Pomposiello et al., 2001).

In both of the above cases, the transcription of *zwf* and *sodA* are known to be directly influenced by the regulators MarA, SoxS, and Rob (Martin and Rosner, 2002). As abundantly clear in the preceding discussions, MarA, SoxS, and Rob are capable of regulating a large number of genes involved in the intrinsic multiple antibiotic resistance response. Numerous studies have employed transcriptional microarrays and transposon mutagenesis to better understand the genetic targets of MarA, SoxS, and Rob (Bennik et al., 2000, Ruiz and Levy, 2010, Pomposiello et al., 2001, Barbosa and Levy, 2000, Martin and Rosner, 2002). The following sections will provide an overview of the *marRAB*, *soxRS*, and *rob* genetic systems.

1.4 The *marRAB* genetic system

Discovered by Stuart Levy and coworkers during genetic screens for increased tolerance to the antibiotic tetracycline, the *marRAB* (multiple antibiotic resistance) operon has served as a model system in understanding regulated intrinsic antibiotic resistance in *E. coli* and related enteric γ-proteobacteria (George and Levy, 1983a, George and Levy, 1983b). The *marRAB* operon encodes genes for three proteins: MarR, MarA, and MarB. MarR and MarA are both DNA-binding, transcriptional regulatory proteins, while MarB is a protein of unknown function.

MarR and MarA are known to negatively and positively regulate, respectively, transcription of the P_{marRAB} promoter and thereby their own expression (Ariza et al., 1994, Martin et al., 1996, Martin et al., 1995, Martin and Rosner, 1995). Induction of *mar*-dependent antibiotic resistance is mediated by the expression of MarA which regulates numerous genes involved in the intrinsic resistance phenotype (Cohen et al., 1993a, Barbosa and Levy, 2000, Gambino et al., 1993).

As a foundational member of a large class of bacterial, DNA-binding proteins, MarR represses the expression of the *marRAB* operon through direct binding of two sites in the *marRAB* promoter (Martin and Rosner, 1995). Functional analysis and crystal structural evidence have demonstrated that MarR contains a conserved helix-turn-helix DNA binding motif and binds to palindromic and pseudopalidromic operator sequences as a homodimer (Alekshun et al., 2001, Alekshun et al., 2000, Martin and Rosner, 1995).

Like many of its homologues, MarR is capable of binding small molecules. Specifically, MarR has been observed bind salicylic acid, a weak aromatic acid, resulting in a loss of DNA binding ability and increased transcription of the *marRAB* operon (Martin and Rosner, 1995). Other studies have demonstrated chemicals such as menadione and plumbagin are capable of altering the DNA binding ability of MarR (Alekshun and Levy, 1999a). In addition to the binding of small molecules, MarR has recently been observed to be bound by two proteins transketolase A (TrkA) and DNA gyrase A (GyrA) in *E. coli* (Domain et al., 2007,

Domain and Levy, 2010). Both enzymes have been implicated in the superoxide stress response, therefore, Domain and coworker have hypothesized that this enzyme serves a regulatory role in activating *marRAB* expression during oxidative stress. Genetic and biochemical evidence suggests the binding of these proteins to MarR causes a decrease in the ability of MarR to bind the *marRAB* promoter and subsequent increased expression of MarA (Domain and Levy, 2010, Domain et al., 2007).

While MarR negatively regulates *marRAB* transcription, MarA is an activator of the *marRAB* operon and thus an autoactivator of its own expression (Martin et al., 1996). Likewise, MarA is directly involved in the positive and negative regulation of numerous downstream genetic targets involved in intrinsic resistance (Jair et al., 1995, Martin et al., 2008, Schneiders and Levy, 2006, Wall et al., 2009). At just over 15 kDa, MarA is a small regulatory protein and is a member of the AraC/XylS family of transcriptional regulators. Similar to other AraC/XylS family proteins, MarA contains two helix-turn-helix DNA binding domains (Rhee et al., 1998). Unlike many AraC/XylS regulators which often function as dimers, MarA binds to promoter regions as a monomer (Jair et al., 1995).

Of the AraC/XylS regulatory proteins, MarA was the first to be fully crystallized in contact with DNA (Rhee et al., 1998). As result, extensive genetic and biochemical analysis has been performed to understand its involvement in

promoter activation and repression. Through biochemical and bioinformatic analysis, MarA is known to bind to a degenerate 19 base-pair sequence known as the *marbox* (AYNGCACNNWNNRYYAAAY) (Martin et al., 1999). In promoters regulated by MarA, the *marbox* has been found in only a few arrangements with respect to the -10 and -35 promoter regions (Martin et al., 1999). Like other transcription factors such as CRP, MarA is able to interact with RNA polymerase in an ambidextrous fashion (Jair et al., 1996a). By ambidextrous, it is meant that MarA can interact with RNA polymerase in two different ways depending on the arrangement of mar-box sites in the promoter creating two classes of promoters. For class I promoters, MarA binds to a *marbox* site upstream of the -35 region and requires contact with the C-terminal domain of the RNA polymerase α-subunit (α-CTD) to activate or repress promoters. Conversely, in class II promoters MarA binds to *marbox* sequences overlapping the -35 region contact with the α-CTD is not required (Schneiders and Levy, 2006).

An additional feature of MarA interaction with promoter regions is that it is capable of activating or repressing transcription depending on its orientation and the location of the *marbox* site (Schneiders and Levy, 2006). In the above cases, the result of MarA interaction is the activation of transcription. For cases of transcriptional repression, a similar classification has been adopted. Type I repression occurs when MarA binds upstream of the -35 region and the N-terminus contacts RNAP in a manner similar to class II activation. Unlike any of the prior cases, Type II repression is mediated by MarA binding between the -10

and -35 regions in a promoter, resulting in the occlusion of RNA polymerase binding, similar to the classical transcriptional repressor model. The net result is that the degeneracy of DNA binding and ambidextrous nature of interaction with RNA polymerase in promoters allows the targets of MarA regulation to be broad. This fact is directly reflected in the large number of genes regulated by MarA.

Apart from its interaction with DNA, MarA is itself regulated at the protein level. MarA has been found to be rapidly degraded by the ATP-dependent Lon protease (Griffith et al., 2004). Based on these findings it has been proposed that rapid degradation of MarA allows for MarA-dependent regulation to be quickly silenced once an appropriate chemical stress has been removed. Additionally, overexpression of MarA is known to result in loss of cellular viability (Griffith et al., 2004). It stands to reason then that rapid degradation of MarA may be a mechanism to prevent cytotoxicity caused by MarA.

1.5 The *soxRS* system

The *soxRS* (superoxide) system is a model system in understanding intracellular redox sensing in bacteria. Initially discovered by screens looking for regulators of *nfo* (Endonuclease IV) and mutants with increased tolerance to redox cycling compounds (Greenberg et al., 1990, Tsaneva and Weiss, 1990), the *soxRS* system encodes two DNA binding transcription factors, SoxR and SoxS. Both of these transcription factors are essential for the effective response to intracellular

superoxide generation. On the chromosome, the *soxR* and *soxS* genes are oriented and transcribed divergently to one another.

SoxR is a $2Fe^{2+}$-2S (iron-sulfur cluster) containing transcription factor of the MerR family of DNA binding proteins (Hidalgo et al., 1995, Hidalgo and Demple, 1994, Hidalgo and Demple, 1996). In solution, SoxR exists as a dimer with each monomer containing a single $2Fe^{2+}$-2S cluster (Hidalgo et al., 1995, Hidalgo and Demple, 1994). On oxidation of the iron-sulfur cluster, SoxR activates the P_{soxS} promoter resulting in upregulated expression of SoxS (Hidalgo et al., 1995, Hidalgo and Demple, 1994). The DNA binding affinity of SoxR is unaffected by its oxidation state, and therefore it is believed that SoxR remains bound to the *soxRS* control region at all times (Hidalgo et al., 1995, Hidalgo and Demple, 1996). A unique feature of SoxR in *E. coli* is that it is only known to regulate a small number of genes outside of *soxS* (Fuentes et al., 2001). Other SoxR homologues in species such as *Pseudomonas putida, Pseudomonas aeruginosa* and *Streptomyces coelicolor* have been observed to possess extensive downstream regulons and to not work through another transcription factor such as SoxS (Dietrich et al., 2008, Park et al., 2006).

SoxS is a homolog of MarA and its properties in transcriptional regulation are nearly identical (Fawcett and Wolf, 1994, Jair et al., 1996a, Wood et al., 1999). Additionally, SoxS binds DNA as a monomer (Wood et al., 1999). Like MarA, SoxS is targeted for rapid degradation by Lon protease which is believed

to be a mechanism for rapid SoxS inactivation (Griffith et al., 2004). Also, SoxS is known to negatively autoregulate its own expression through direct interaction with the P_{soxS} promoter (Nunoshiba et al., 1993b). Although MarA and SoxS do possess highly overlapping downstream regulons, it has been observed the relative affinity of these regulators for similar promoters is not equal resulting in differential transcriptional activation and repression (Martin et al., 2000).

The specific signal for oxidation of SoxR is not entirely clear, although it is known that intracellular superoxide and nitric oxide species can trigger SoxR activation *in vivo* (Greenberg et al., 1990, Nunoshiba et al., 1993a). Recently, Lee and coworkers have shown that electrons can be withdrawn from DNA-bound SoxR to oxidized guanidine residues thereby oxidizing SoxR and activating SoxS expression (Lee et al., 2009). This implies that in addition to intracellular superoxides, DNA damage may play a larger role in *soxRS* activation.

1.6 The *rob* system

Rob (right origin binding) is a cryptic transcriptional regulator in *E. coli* discovered during a large-scale screen for proteins binding to the chromosomal origin of replication (*oriC*) (Skarstad et al., 1993, Ali Azam et al., 1999). Later studies determined, based on homology to both MarA and SoxS, that Rob is an AraC/XylS-like regulator capable of binding similar DNA sequences as MarA and

SoxS (Ariza et al., 1995, Li and Demple, 1996, Kwon et al., 2000). Although no apparent phenotype is known for a *rob* mutant, it is known, when overexpressed, to produce a similar multidrug resistance phenotype that is observed in mutants that constitutively express MarA or SoxS (Nakajima et al., 1995). Likewise, treatment of cells with chemicals such as decanoate and dipyridyl are known to produce a Rob-dependent resistance phenotype (Griffith et al., 2009, Rosenberg et al., 2003, Rosner et al., 2002). A point of divergence between Rob and Mara/SoxS is that Rob possesses an additional C-terminal domain believed to be involved in the binding of small molecules, not unlike other AraC/XylS-family regulators (Rosner et al., 2002, Kwon et al., 2000).

The DNA binding specificity and interaction with RNA polymerase between Rob and its homologues MarA and SoxS is extremely similar. In fact, numerous members of the *marA* and *soxS* regulons are activated or repressed by Rob (Bennik et al., 2000, Kwon et al., 2000, Ariza et al., 1995, Li and Demple, 1996). Based on this evidence, this larger set of genes under MarA, SoxS and Rob regulation are known as the *mar/sox/rob* regulon.

Like MarA, Rob has been crystallized in contact with DNA (Kwon et al., 2000). Although significant structural similarities exist between Rob and MarA, Rob has been found to contact DNA with only one helix-turn-helix motif whereas MarA does so with two (Kwon et al., 2000, Rhee et al., 1998). In addition, the regulatory properties of Rob *in vivo* do not appear to match those of Rob *in vitro*

– a point that will be further clarified. Specifically, Rob is expressed constitutively at high levels (~10,000 copies/cell) and remains inactive in absence of chemical stress whereas purified Rob is capable of activating transcription in the absence of any known chemical inducers (Li and Demple, 1996, Ariza et al., 1995, Skarstad et al., 1993, Ali Azam et al., 1999, Jair et al., 1996b).

Where Rob is most markedly different from MarA and SoxS concerns its regulation. For both MarA and SoxS, their expression is regulated at the transcriptional level. Rob, on the other hand, is constitutively expressed and is activated post-translationally (Rosner et al., 2002, Rosenberg et al., 2003, Griffith et al., 2009). Interestingly, recent work by Griffith and coworkers has shown that Rob regulates itself by a 'sequestration-dispersion' mechanism (Griffith et al., 2009). In the inactive state, Rob has been observed to form aggregrated clusters in the cytoplasm. On treatment with known Rob inducers such as decanoate and dipyridyl, Rob becomes homogenously dispersed throughout the cytoplasm. In elegant experiments with SoxS-Rob chimeras, Griffith and coworkers were also able to demonstrate that the 'sequestration-dispersion' mechanism is dependent on the C-terminal domain of Rob. Based on these results, understanding the exact role of small molecule binding and possible other factors involved in Rob activation remains a continued point of interest.

1.7 Defining the *mar/sox/rob* regulon: beyond just genetic targets.

The role of *mar/sox/rob* in cellular physiology. Numerous studies have defined an extensive list of regulatory targets that collectively define the *mar/sox/rob* regulon. Although many of these genes can be ascribed a discernable function for antibiotic resistance, many cannot (Martin and Rosner, 2002). An attractive hypothesis, based on the targets that are well-characterized such as *micF*, *sodA*, or *acrAB*, might be that these systems have evolved for the purpose of responding to toxic environments such as those found in the presence of antibiotic producing soil bacteria. While this may in fact be true, limited data exists to fully support this hypothesis. An alternative perspective may be that these systems have evolved to combat intracellular problems that develop during the metabolism of diverse carbon sources or in certain nutrient limited conditions (Helling et al., 2002, Rosner and Martin, 2009, Piddock, 2006).

A key piece of evidence for the latter hypothesis comes from the recent work of Rosner and Martin who demonstrated that the *mar/sox/rob* regulon is upregulated in mutants lacking the outer membrane pore, TolC (Rosner and Martin, 2009). The authors conclude, based on their analysis, that the accumulation of intermediary metabolites is likely the source of gratuitous inducers of the *marRAB*, *soxRS*, and *rob* systems. In this dissertation, this analysis has been extended to look at a small number of aromatic metabolites as possible inducers, where it will be shown that these can serve as inducers of the

marRAB system. The general perspective of the following work will attempt to remain unbiased in the interpretation of results based on these differing opinions.

The role of *mar/sox/rob* in pathogenesis. An additional point interest for the *mar/sox/rob* network is its potential role in pathogenesis. Early assessments, based on work performed in *Salmonella enterica* serovar Typhimurium, has concluded that neither *marRAB* or *soxRS* are required for infection and colonization in a BALB/c murine model (Sulavik et al., 1997, Fang et al., 1997). Although these findings are indicative of the non-essentiality of MarA, SoxS, and Rob during intestinal infection, conflicting evidence exists for *E. coli* infection models. In particular, work by Casaz and coworkers has shown that mutants in *marRAB*, *soxRS*, and *rob* have attenuated virulence phenotypes in a murine urinary tract infection model (Casaz et al., 2006). These conflicting results underpin the need to understand the physiological and environmental factors in which the *mar/sox/rob* networks have evolved to respond.

1.8 Conclusions

This overview has brought to light the extensive amount of regulatory and biochemical information that is known about the *mar/sox/rob* regulon. Although, many details have been recorded, a shortcoming in our understanding of the *mar/sox/rob* regulon is an integrated, testable regulatory model. Through the use

of the model developed in the early portions of this work, the remaining chapters set out to use this model to answer a number of questions regarding *mar/sox/rob* regulon management and how the coordinated regulatory action of these systems is necessary for an appropriate antibiotic resistance response.

1.9 Figures and Tables

Table 1.1. Genes know to be part of the *mar/sox/rob* regulon. Compiled from the EcoCyc Database (Keseler et al., 2009).

	Known regulon member		
Gene	***marRAB***	***soxRS***	***Rob***
ybaO	X		
acrAB	X	X	X
nfsA		X	
nfsB	X		
fur		X	
ybiS			X
fldA		X	
marRAB	X	X	X
poxB	X	X	
fumC	X	X	X
pqiAB	X	X	
putA	X		
ptsG		X	
zwf	X	X	X
nfo	X	X	X
micF	X	X	X
ribA		X	

Table 1.1 (Continued)

inaA	X	X	X
zinT		X	
tolC	X	X	X
slp	X		
mltF			X
fldB		X	
hdeAB	X		
waaY	X	X	
sodA	X	X	X
aslB			X
fpr	X	X	
purA	X		
rob	X		
pgi		X	

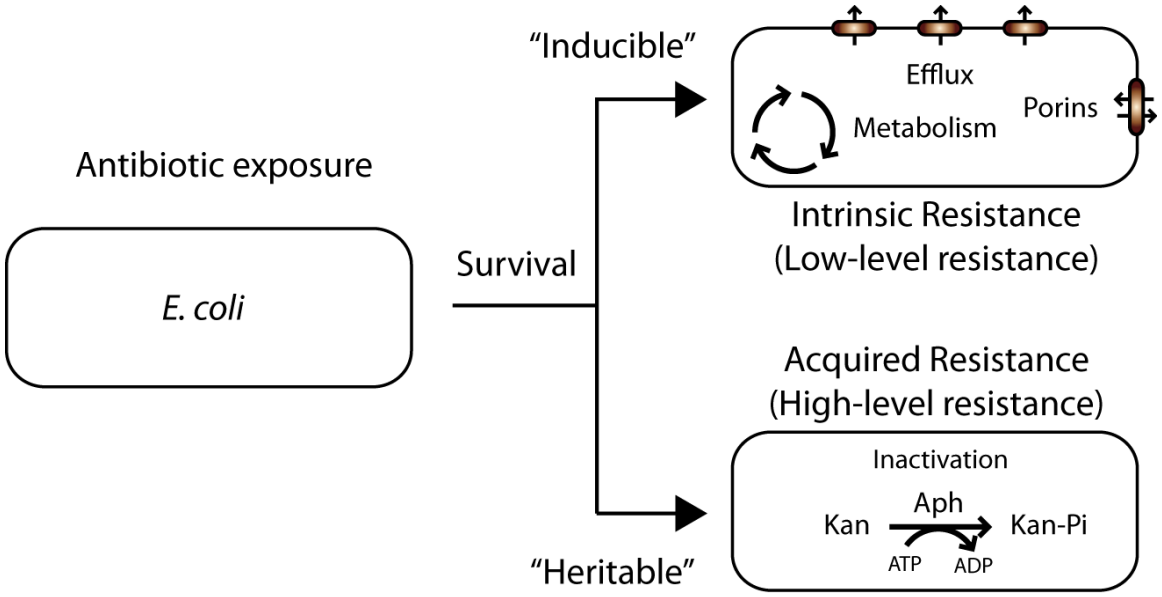

Figure 1.1. Defining differences in intrinsic ("inducible") and acquired ("heritable") forms of antibiotic resistance.

Chapter 2: Materials and Methods

2.1 Bacterial strains, media, and growth conditions

All bacterial strains in this work are isogenic derivatives of *Escherichia coli* K-12 strain MG1655 or MC4100 and may be found in Table 2.1 with respective genotypes. For all propagation and genetic manipulations, strains were routinely grown in Luria-Bertrani (LB) media (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl with 15 g/l bacto agar for solid media) (Miller, 1992). Strains transformed with plasmid or linear DNA by electroporation or chemically induced competence were recovered in super optimal broth with catabolite repression (SOC) media (20 g/l tryptone, 5 g/l yeast extract, 0.5 g/l NaCl, 0.186 g/l KCl, 0.952 g/l $MgCl_2$, 20 mM glucose) (Miller, 1992). Unless otherwise indicated all experiments in this work were conducted in 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffered minimal media (40 mM MOPS, 4 mM tricine, 9.5 mM NH_4Cl, 0.276 mM K_2SO_4, $5x10^{-4}$ mM $CaCl_2$, 0.525 mM $MgCl_2$, 50 mM NaCl, with micronutrients) using the formulation described by Neidhardt and coworkers supplemented with 20 mM glucose and 0.2% casamino acids as carbon sources with a final pH of 7.2, tryptone broth (10 g/l tryptone, 8 g/l NaCl), or Medium A (7g/l nutrient broth, 1 g/l yeast extract, 2 g/l glycerol, 3.7 g/l K_2HPO_4, 1.3 g/l KH_2PO_4) (Kawaji et al., 1979, Neidhardt et al., 1974, Miller, 1992). Strains were grown at 37^{o}C with the exception of those harboring temperature-sensitive plasmids such as pKD46, pCP20, pINT-ts, or pAH123. Curing strains of these plasmids was typically

performed by overnight growth on solid LB media without antibiotic selection at 42^{o}C. Antibiotics were used at the following concentrations for plasmid propagation: 100 µg/ml ampicillin, 40 µg/ml kanamycin, and 20 µg/ml chloramphenicol. For chromosomal selection antibiotics were used the following concentrations: 20 µg/ml kanamycin, 10 µg/ml chloramphenicol, and 12.5 µg/ml tetracycline. All experiments using strains with chromosomal insertions or deletions were conducted in the absence of antibiotic selection.

2.2 Reagents and chemical preparation

Reagents and chemicals used in this study were purchased from Sigma-Aldrich. Where possible, chemicals were dissolved in water and stored at -20^{o}C. Stock concentrations of sodium salicylate (Sigma, S3007) and paraquat (methyl viologen dichloride hydrate) (Aldrich, 856177) were 0.5M and 50 mM, respectively. Due to poor solubility, sodium decanoate (Sigma, C4151) was supplemented to concentrated aliquots of inducing media immediately prior to use. Other organic acids used that were unavailable as sodium salts were brought into aqueous solution by titration with 5M NaOH until a pH of 7.0. Several aromatic acid solutions were subject to rapid oxidation and were prepared immediately prior to use to avoid prolonged storage. Nutritional supplements such as indole (Aldrich, I3408) were readily dissolved in dimethyl sulfoxide (DMSO).

2.3 Strain and plasmid construction

All targeted insertions and deletions created in this work were constructed using the λ-Red recombinase method of Datsenko and Wanner (Datsenko and Wanner, 2000). Briefly, deletion cassettes containing 40 base-pair (bp) regions of homologous DNA to sites on the chromosome were amplified by polymerase chain reaction (PCR). These products were purified using standards PCR clean-up kits (Qiagen) or ethanol precipitation. Strains harboring the λ-Red recombinase expression vector, pKD46, were grown at 30^{o}C in LB or SOB, induced with 0.2% arabinose, and made electrocompetent. DNA was transformed into these cells using an electroporator (BIO-RAD) (5 ms, 2.5 mV). Transformed cells were recovered for 2 to 4 hrs at 37^{o}C in SOB media followed by plating on selective LB solid media. All deletions were transduced into their parent background, typically MG1655, using generalized P1*vir* transduction.

All plasmids were constructed using standard methods (Sambrook, 2001). All PCR amplifications were made using MG1655 DNA and Phusion polymerase (New England Biolabs). Amplified DNA and vector DNA were treated with restriction endonuclease enzymes as per manufacturer's instructions (New England Biolabs). Ligated clones were transformed into *recA* hosts DH5α, DH5αZ1, or BW25141. Successful clones were verified by diagnostic PCR and DNA sequencing.

Single-copy transcriptional and translational fusions to YFP were made using the pVenus vector (Saini et al., 2009). All promoter fragments were cloned into the EcoRI and KpnI sites of pVenus. Subsequent clones were transformed into MG1655 harboring the pINT-ts helper plasmid to express Intλ and promote site specific recombination of the pVenus derivatives into the attλ site on the *E. coli* chromosome. Single-copy fusions were then transferred to other strains by P1*vir* generalized transduction.

2.4 Protein purification

Transcriptional regulatory proteins purified for *in vitro* assays were prepared by affinity chromatography using protein-protein fusions to either glutathione-S-transferase (GST), 6xHistidine (6xHis) or 6xHistidine-Asparagine (6xHN) tags. Unless otherwise specified, proteins were purified using a ÄKTA Prime fast protein liquid chromatograph (FPLC) (GE Healthcare). Explicit procedures for individual proteins will be described in the following sections.

Purification of MarR. To purify the GST-MarR fusion protein, BL21(DE3) cells harboring pGEX-marR were grown in 2-liter LB cultures at 37^{o}C with 250 rpm shaking until an OD=0.7 as determined using a UV-1800 spectrophotometer (Shimadzu). Expression of GST-MarR was then induced by the addition of IPTG to 1 mM, followed by an additional 4 hrs of growth at 37^{o}C. Cells were collected

and pelleted by centrifugation at 7000 x *g* for 10 minutes and were stored overnight at -80^{o}C. The cell pellets were then resuspended in 3 ml of tris-buffered saline (TBS) (50 mM Tris, 150 mM NaCl, pH = 7.4) plus 1% Triton X-100 and 1 mM dithiothreitol (DTT) for every 1 g of cell pellet. Resuspended cells were then disrupted by sonication (8 x 10s pulse). Cell debris was cleared by centrifugation at 10,000 x *g* for 10 minutes followed by 40,000 x *g* for 1 hr.

Clarified cell lysate was then loaded onto a 5-ml GSTrap column (GE Healthcare) pre-equilibrated with TBS. The column was then washed with 5 bed volumes of TBS followed by elution with 20 ml of glutathione elution buffer (50 mM Tris, 10 mM glutathione, pH = 8.0). The GST-MarR fusion was then dialyzed against 8 x 600 ml changes of PreScission protease buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, pH = 8.0). The GST tag was then removed by proteolytic cleavage using 100 units of PreScission protease (GE Healthcare) for 12 h at 4^{o}C. The digested solution was then passed over a GSTrap column pre-equilibrated with TBS to remove the cleaved GST and PreScission protease. The flow-through containing purified MarR was collected and dialyzed against 8 x 600 ml of TBS. Protein concentration was determined using the bicinchoninic acid (BCA) reagent assay (Pierce) using bovine serum albumin (BSA) standards. Aliquot were frozen at -80^{o}C and stored until use.

Purification of Rob. To purify the GST-Rob fusion, a nearly identical protocol to that described for GST-MarR was used with noted exceptions. The GST-Rob fusion was expressed from pGEX-rob in BL21(DE3) as outlined for GST-MarR. For purification steps, all buffers (TBS and GEB) were formulated with 0.5M NaCl. Likewise, after cleavage of GST from Rob by PreScission protease digestion and subsequent removal of GST and protease, Rob was dialyzed extensively (10 x 600 ml) a high salt storage buffer (50 mM Tris-HCl, 0.5 M NaCl, 1 mM DTT, 20% glycerol, pH=8.0) and stored at -80^{o}C. For experiments conducted in the presence of MarA, Rob was transferred into Buffer A (50 mM HEPES, 1 M NaCl, 1 mM DTT, 5 mM EDTA, 0.1% Triton X-100, pH=8.0) using a Micro Bio-Spin P-6 (BIO-RAD) buffer exchange column. Final proteins were determined using the BCA assay method using BSA standards after tricholoracetic acid (TCA) precipitation.

Purification of MarA. Purification of MarA protein was performed using Ni-affinity chromatography under batch, denaturing conditions in a manner similar to Jair and coworkers (Jair et al., 1995). MarA was expressed with a N-terminal 6xHis tag from pET28a-marA in BL21(DE3). Cells were grown in 200 ml of LB media at 37^{o}C and 250 rpm shaking to an OD=0.7 as measured using an UltroSpec 10 spectrophotometer (Amersham) followed by induction with 0.5 mM IPTG. Cultures were grown for an additional two hours, followed by centrifugation

at 4000 rpm for 20 minutes at 20^{o}C. The pellet was then washed once with phosphate buffered saline (PBS) (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 138 mM NaCl, 2.7 mM KCl, pH=7.4), and repelleted The cell pellet was then frozen at -80^{o}C before any further steps.

Following cryogenic freezing, the pellet was thawed on ice and gently resuspended in 5 ml of Buffer B (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8M Urea, pH=8.0) per gram of cell pellet. The cell suspension was then gently vortexed for 5 minutes followed by incubation at room temperature for an additional 60 minutes. Cell lysis was determined complete once the solution became translucent and viscous. Lysate was then clarified by centrifugation at 6,000 x *g* for 30 minutes. The clarified lysate was then transferred to a screw-cap 5 ml column, followed by the addition of 1 ml of 50% Ni-NTA resin (Qiagen) slurry per 5 ml of cell extract that had been washed to remove storage ethanol and preincubated in Buffer B for 10 minutes. The mixture was then incubated at room temperature for 60 minutes with gentle agitation on a platform shaker to ensure uniform binding of 6xHis-MarA.

Following incubation, the Ni-NTA resin was allowed to settle and form a bed free of air bubbles in the column. The cell lysate was subsequently drained and collected from the column by gravity flow through the resin. After this step, the Ni-NTA resin was washed twice with 10 bed volumes of Buffer C (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8M urea, pH=6.3). To remove non-specifically bound

products, the resin was washed four times with one bed volume of Buffer D (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8M urea, pH=5.9). Finally, denatured MarA was collected by acidic elution with four bed volumes of Buffer E (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8M urea, pH=4.7). MarA protein was then renatured by extensive (8 x 1 L), slow dialysis against Buffer A (50 mM HEPES, 1 M NaCl, 1 mM DTT, 5 mM EDTA, 0.1% Triton X-100, pH=8.0) at 4^{o}C. After renaturing, non-soluble precipitate was removed by centrifugation at 10,000 x *g* for 30 minutes. Renatured 6xHis-MarA was then concentrated 5X with a 10,000 MWCO centrifugal concentrator (Amicon).

Finally, the 6xHis tag was removed by digestion overnight at 4^{o}C with Thrombin-argarose beads (RECOMT, Sigma-Aldrich) as per manufacturers instructions. Thrombin-agarose beads were removed by column filteration. Digested MarA was then dialyzed against Buffer A (6 x 1L) in a 8,000 MWCO membrane to remove the thrombin reaction buffer components and the cleaved 6xHis tag. Complete removal of the tag was verified by SDS-PAGE and Coomassie staining. Final protein concentrations were determined by the BCA assay method using BSA standards after TCA precipitation.

Purification of 6xHN-CRP. Purification of 6xHN-CRP was performed using Co-affinity chromatography under batch, native conditions. CRP was expressed with a N-terminal 6xHN tag from pPROTet.E-crp-1 in LC100. Cells were grown in 50

ml of LB media at 37^{o}C and 250 rpm shaking to an OD=0.7 as measured using an UltroSpec 10 spectrophotometer (Amersham) followed by induction with 100 µg/ml anhydrotetracycline (aTc). Cultures were grown for an additional three hours, followed by centrifugation at 4000 rpm for 20 minutes at 20^{o}C. The pellet was then washed once with phosphate buffered saline (PBS) (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 138 mM NaCl, 2.7 mM KCl, pH=7.4), and repelleted. The cell pellet was then frozen at -80^{o}C before any further steps.

Cell pellets thawed and resuspended in 5 ml of TBS (50 mM Tris, 150 mM NaCl, pH=7.4) with 0.1% Triton X-100 per gram of cells. Resuspended cells were then disrupted by sonication (8 x 10s pulses). Cell debris was cleared by centrifugation at 10,000 x *g* for 30 minutes. The clarified extract was then incubated at 4^{o}C for 1 hour with 1 ml of 50% TALON resin slurry (Clontech) that had been thoroughly washed and preincubated with TBS. Incubation was performed with gentle agitation on a platform shaker.

Following incubation, the TALON resin mixture was transferred to a 5 ml spin column and allowed to settle and form a bed free of air bubbles in the column. The cell lysate was subsequently drained and collected from the column by gravity flow through the resin. Following this step, the resin was washed with 5 bed volumes of wash buffer (50 mM Tris, 150 mM NaCl, 10 mM imidazole, pH=7.4) and eluted with 4 bed volumes of elution buffer (50 mM Tris, 150 mM NaCl, 150 mM imidazole, pH = 7.4). The eluted protein was then concentrated

5X with a 10,000 MWCO concentrator cassette (Amicon) followed by dialysis against CRP storage buffer (50 mM Tris, 100 mM KCl, 1 mM DTT, 1 mM EDTA, 5% glycerol) . Final protein concentrations were determined by the BCA assay method after TCA precipitation.

2.5 Electromobility shift assays

Electromobility shift assays were conducted using a 150 basepair fragment of the *marRAB* promoter or a 186 base-pair fragment of the *lac* promoter generated by PCR using primers LC468 and LC257 or LC554 and LC555, respectively. Following PCR purification, DNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific). For labeling reactions, 100 ng of DNA was labeled with 10 µCi of γP^{32}-ATP with T4 polynucleotide kinase (New England Biolabs). Binding reactions were carried out in 20 µl of buffer (50 mM Tris, 1 mM EDTA, 50 mM NaCl, 75 ng/µl Herring Sperm DNA, 10% glycerol, pH=8.0) with indicated concentrations of inducers. For aromatic acids, 50 mM aqueous stock solutions were prepared by slowly buffering solutions to a pH = 7.2 with NaOH. Reactions containing purified protein and labeled DNA were allowed to proceed at room temperature for 15 minutes for MarA, Rob, or 6xHN-CRP binding reactions and 30 minutes for MarR binding reactions. Reaction products were then displayed on prerun 5% 0.5X TBE-buffered, acrylamide gels run at 150 V/10 mA for 30 minutes at 4^{o}C. Gels were then transferred to filter

paper, dried, and imaged using a phosphor screen (Applied Biosystems) and Storm 840 PhosphorImager (Amersham). Image analysis was performed using ImageJ.

2.6 Isothermal titration calorimetry

All experiments were conducted using a MicroCal VP-ITC titration calorimeter preincubated to 25^{o}C for at least one-hour prior to the start of experiments. MarR or Rob protein solutions were brought to a final concentration of 10 µM in TBS (150 mM NaCl for MarR, 500 mM for Rob) and pH was measured (typically between 7.2 and 7.4) using a Perkin-Elmer pH meter. Ligand solutions were prepared fresh in TBS, the pH of the solution was adjusted to that of the MarR or Rob solution, and the final concentration was brought to 10 mM. The 1.4 ml sample well was loaded with a blunt-end needle attached to a 5 ml Hamilton pipette making sure to introduce no air bubbles into the sample cell. Likewise, the injection syringe was filled and expelled with the 10 mM ligand solution twice prior to finally being filled and made free of any air bubbles. Experimental parameters used with the VP-ITC were 28 x 10 µl injections with 5 minute spacings, 300 rpm stirring speed, and a reference power of 1 µcal/s.

2.7 Outer membrane protein preparation and display

Outer membrane preparations were performed as described by Slauch and Silhavy (Slauch and Silhavy, 1989). Cells were grown overnight in Medium A, and subcultured 1:200 in 10 ml of fresh Medium A in 20 x 150 mm glass test tubes and grown at 37^{o}C with 250 rpm shaking. High osmolarity conditions were created by increasing the NaCl concentration in Medium A to 200 mM. Cultures were grown to an OD=0.4 as determined by an UltroSpec 10 spectrophotometer (GE Healthcare) with 1 cm path length cuvettes.

Cultures were centrifuged at 4000 rpm for 20 minutes. The cell pellet was resuspended once in 10 ml of 30 mM Tris-HCl (pH=8.1), and centrifuged again for 10 minutes. The supernatant was thoroughly decanted and pellets were frozen at -80^{o}C for at least 30 minutes. Following cryogenic freezing, the pellet was thawed and gently resuspended in 0.2 ml of 30 mM Tris-HCl/20% sucrose (pH=8.1), and placed on ice. Then, 20 µl of 10 mg/ml lysozyme (in 100 mM EDTA, pH=7.1), was added to the suspension, gently mixed, and allowed to incubate on ice for 30 minutes. Following lysozyme treatment, 1.5 ml of 3 mM EDTA (pH=7.1) was added, and the resulting spheroblasts were sonicated with a microtip sonicator using one, 20s pulse at room temperature.

The homogenized lysate (1.5 ml) was then centrifuged at 14,000 rpm for 60 minutes. The supernatant was carefully decanted as to not dislodge the pelleted, insoluble membrane fraction. Membrane pellets were then resuspended

in 20 µl of Laemelli SDS sample buffer and boiled for 5 minutes at 100^{o}C. The samples were then loaded and displayed on a 10% acrylamide:bisacrylamide (37.5:1) Tris Buffered SDS/6M urea gel run at 150V for 75 minutes at room temperature. Gels were fixed and stained with Coomassie Brilliant Blue R-250 (Sigma) and scanned with a Hewlett Packard desktop scanner. Images were processed and analyzed using ImageJ.

2.8 Transcriptional reporter assays

96-well growth conditions. For the purposes of uniform growth, induction time and reporter expression assays, a deep well plate assay procedure was developed based on the assay of Griffith and Wolf (Griffith and Wolf, 2002) with modifications. Replicate overnight cultures were grown in 2 ml of media in 16x100 mm glass test tubes. Each culture was subcultured in duplicate 1:200 after which 0.45 ml was transferred to a single well of a polypropylene, 2.2 ml, deep, square, 96-well microtiter plate (VWR Scientific,). The plates were then sealed with Breathe-Easy membranes (Sigma, Z380059) to reduce media evaporation and allow gas transfer for uniform aeration. Plates were subsequently placed on a high-speed microtiter plate shaker and agitated at 1000 rpm at 37^{o}C. Typical assays involved growth of cells to an OD=0.5 as determined using an UltroSpec 10 spectrophotometer (GE Healthcare). At this time 100 µl of media containing media with or without dissolved inducer was

rapidly added using a multichannel micropipettor. Following induction, plates were resealed with Breathe-Easy membrane and allowed to continue incubating at 1000 rpm and 37^{o}C for an additional hour prior to measurements.

Fluorescence measurements. Fluorescence and optical density measurements were made with a Tecan Safire2 plate reader. All measurements were made with a gain set at 125 and 515/530 nm (ex/em) filter setting for YFP fluorescence and 440/480 nm (em/ex) for CFP fluorescence. Absorbance was set of 600 nm for optical density measurements. For cultures grown in MGC or Medium A 250 µl of culture was used for measurements. For cultures grown in LB, 150 µl of culture was used for measurements.

2.9 Tables

Table 2.1. Bacterial strains used in this work.

Strain	Genotype	Reference
MG1655	*F⁻ λ⁻ rph-1 rfb-50*	CGSC
MC4100	*F- λ- [araD139] Δ(lacZYA-argF)U169 e14- flhD5301 Δ(fruK-yeiR)725(fruA25) relA1 rpsL150(StrR) rbsR22 Δ(fimB-finE)632(::IS1) deoC1*	CGSC
DH5α	*F- λ- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169 hsdR17*	Lab stocks
DH5αZ1	*F- λ- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169 hsdR17 attλ::(spcR lacIq tetR)*	(Lutz and Bujard, 1997)
BW25141	*F- λ- Δ(araB-araD)567 ΔlacZ4787(::rrnB-3) Δ(phoB-phoR)580 galU95 ΔuidA3::pir+ recA1 ΔendA9::FRT rph-1 Δ(rhaB-rhaD)568 hsdR514*	CGSC, (Haldimann and Wanner, 2001)
BL21(DE3)	*F- ompT gal dcm lon hsdS(rb-,rm-) λ(DE3 [lacI lacUV5-T7 gene1 ind1 sam7 nin5])*	Lab stocks
JW5249	*F- λ- rph-1 Δ(araB-araD)567 ΔlacZ4787(::rrnB-3) Δ(rhaB-rhaD)568 hsdR514 marA752::kan*	CGSC, (Baba et al., 2006)
JW4023	*F- λ- rph-1 Δ(araB-araD)567 ΔlacZ4787(::rrnB-3) Δ(rhaB-rhaD)568 hsdR514 soxS756::kan*	CGSC, (Baba et al., 2006)
JTG1078	*F⁻ λ⁻ rph-1 rfb-50 INV(rrnD-rrnE)1 rpsL179 soxR105 zjc-2206::Tn10dKan*	CGSC, (Nunoshiba and Demple, 1994)
MDG147	Φ(*ompF⁺-yfp⁺*)*30* Φ(*ompC⁺-cfp⁺*)*31*	(Batchelor et al., 2005)
LC100	*F⁻ λ⁻ rph-1 rfb-50 attλ::(spcR lacI^q $P_{T5/N25}$-tetR)*	(Chubiz and Rao, 2008)
LC310	*Δrob::kan*	
LC311	*ΔsoxRS::kan*	
LC312	*ΔmarRAB::kan*	
LC317	*ΔmarRAB::cm ΔsoxRS::kan*	
LC318	*Δrob::kan ΔsoxRS::cm*	
LC319	*Δrob::kan ΔmarRAB::cm*	
LC320	*Δrob::FRT*	
LC321	*ΔsoxRS::FRT*	
LC322	*Δrob::FRT ΔsoxRS::FRT*	
LC433	*attλ::mar-'venus*	
LC434	*attλ::inaA-'venus*	
LC439	*attλ::micF-'venus*	
LC440	*attλ::rob-'venus*	
LC441	*attλ::soxS-'venus*	
LC442	*attλ::tolC-'venus*	
LC454	*ΔsoxS756::kan*	
LC455	*ΔsoxS756::kan ΔmarA752::FRT*	
LC456	*ΔsoxS756::kan Δrob::FRT*	
LC457	*ΔsoxS756::kan ΔmarA752::FRT Δrob::cm*	
LC458	*ΔsoxS756::FRT*	
LC459	*ΔsoxS756::FRT ΔmarA752::FRT*	
LC460	*ΔsoxS756::FRT Δrob::FRT*	
LC461	*ΔsoxS756::FRT attλ::venus*	
LC462	*ΔsoxS756::FRT attλ::mar-venus*	
LC463	*ΔsoxS756::FRT attλ::soxS-venus*	
LC464	*ΔsoxS756::FRT attλ::rob-venus*	
LC465	*ΔsoxS756::FRT attλ::inaA-venus*	
LC466	*ΔsoxS756::FRT Δrob::FRT attλ::venus*	
LC467	*ΔsoxS756::FRT Δrob::FRT attλ::mar-venus*	
LC468	*ΔsoxS756::FRT Δrob::FRT attλ::soxS-venus*	
LC469	*ΔsoxS756::FRT Δrob::FRT attλ::rob-venus*	
LC470	*ΔsoxS756::FRT Δrob::FRT attλ::inaA-venus*	
LC471	*ΔmarA752::FRT*	
LC472	*ΔmarA752::FRT ΔsoxS756::FRT*	
LC473	*ΔmarA752::FRT Δrob::FRT*	
LC474	*ΔmarA752::FRT ΔsoxS756::FRT Δrob::FRT*	
LC475	*ΔmarA752::FRT attλ::mar-'venus*	
LC476	*ΔmarA752::FRT attλ::soxS-'venus*	
LC477	*ΔmarA752::FRT attλ::rob-'venus*	
LC478	*ΔmarA752::FRT attλ::inaA-'venus*	

Table 2.1 (Continued)

LC479	*ΔmarA752::FRT ΔsoxS756::FRT attλ::mar-'venus*
LC480	*ΔmarA752::FRT ΔsoxS756::FRT attλ::soxS-'venus*
LC481	*ΔmarA752::FRT ΔsoxS756::FRT attλ::rob-'venus*
LC482	*ΔmarA752::FRT ΔsoxS756::FRT attλ::inaA-'venus*
LC483	*ΔmarA752::FRT Δrob::FRT attλ::mar-'venus*
LC484	*ΔmarA752::FRT Δrob::FRT attλ::soxS-'venus*
LC485	*ΔmarA752::FRT Δrob::FRT attλ::rob-'venus*
LC486	*ΔmarA752::FRT Δrob::FRT attλ::inaA-'venus*
LC487	*ΔmarA752::FRT ΔsoxS756::FRT Δrob::FRT attλ::mar-'venus*
LC488	*ΔmarA752::FRT ΔsoxS756::FRT Δrob::FRT attλ::soxS-'venus*
LC489	*ΔmarA752::FRT ΔsoxS756::FRT Δrob::FRT attλ::rob-'venus*
LC490	*ΔmarA752::FRT ΔsoxS756::FRT Δrob::FRT attλ::inaA-'venus*
LC492	*Δrob::FRT attλ::mar-'venus*
LC493	*Δrob::FRT attλ::soxS-'venus*
LC494	*Δrob::FRT attλ::rob-'venus*
LC495	*Δrob::FRT attλ::inaA-'venus*
LC496	*ΔmarRAB::FRT*
LC497	*ΔmarRAB::FRT ΔsoxRS::FRT*
LC539	*ΔmarRAB::FRT ΔsoxRS::FRT Δrob::FRT*
LC551	*ΔtolC::cat*
LC567	*ΔentCEBAH::cat*
LC614	*ΔtolC::FRT*
LC617	*ΔtolC::FRT Δent::cat*
LC621	*ΔtolC::FRT attλ::(mar'-venus oriR6K kan)*
LC623	*ΔtolC::FRT attλ::(micF'-venus oriR6K kan)*
LC627	*ΔtolC::FRT Δent::FRT*
LC634	*ΔtolC::FRT Δent::FRT attλ::(mar'-venus oriR6K kan)*
LC665	*ΔmarRAB::FRT attλ::(mar'-venus oriR6K kan)*
LC666	*ΔsoxRS::FRT attλ::(mar'-venus oriR6K kan)*
LC667	*Δrob::FRT attλ::(mar'-venus oriR6K kan)*
LC668	*ΔmarRAB::FRT ΔsoxRS::FRT attλ::(mar'-venus oriR6K kan)*
LC669	*Δrob::FRT ΔsoxRS::FRT attλ::(mar'-venus oriR6K kan)*
LC670	*ΔmarRAB::FRT Δrob::FRT attλ::(mar'-venus oriR6K kan)*
LC671	*ΔmarRAB::FRT ΔsoxRS::FRT Δrob::FRT attλ::(mar'-venus oriR6K kan)*
LC672	*ΔmarRAB::FRT attλ::(soxS'-venus oriR6K kan)*
LC673	*ΔsoxRS::FRT attλ::(soxS'-venus oriR6K kan)*
LC674	*Δrob::FRT attλ::(soxS'-venus oriR6K kan)*
LC675	*ΔmarRAB::FRT ΔsoxRS::FRT attλ::(soxS'-venus oriR6K kan)*
LC676	*Δrob::FRT ΔsoxRS::FRT attλ::(soxS'-venus oriR6K kan)*
LC677	*ΔmarRAB::FRT Δrob::FRT attλ::(soxS'-venus oriR6K kan)*
LC678	*ΔmarRAB::FRT ΔsoxRS::FRT Δrob::FRT attλ::(soxS'-venus oriR6K kan)*
LC679	*ΔmarRAB::FRT attλ::(rob'-venus oriR6K kan)*
LC680	*ΔsoxRS::FRT attλ::(rob'-venus oriR6K kan)*
LC681	*Δrob::FRT attλ::(rob'-venus oriR6K kan)*
LC682	*ΔmarRAB::FRT ΔsoxRS::FRT attλ::(rob'-venus oriR6K kan)*
LC683	*Δrob::FRT ΔsoxRS::FRT attλ::(rob'-venus oriR6K kan)*
LC684	*ΔmarRAB::FRT Δrob::FRT attλ::(rob'-venus oriR6K kan)*
LC685	*ΔmarRAB::FRT ΔsoxRS::FRT Δrob::FRT attλ::(rob'-venus oriR6K kan)*
LC686	*ΔmarRAB::FRT attλ::(inaA'-venus oriR6K kan)*
LC687	*ΔsoxRS::FRT attλ::(inaA'-venus oriR6K kan)*
LC688	*Δrob::FRT attλ::(inaA'-venus oriR6K kan)*
LC689	*ΔmarRAB::FRT ΔsoxRS::FRT attλ::(inaA'-venus oriR6K kan)*
LC690	*Δrob::FRT ΔsoxRS::FRT attλ::(inaA'-venus oriR6K kan)*
LC691	*ΔmarRAB::FRT Δrob::FRT attλ::(inaA'-venus oriR6K kan)*
LC692	*ΔmarRAB::FRT ΔsoxRS::FRT Δrob::FRT attλ::(inaA'-venus oriR6K kan)*
LC693	*ΔmarRAB::FRT attλ::(micF'-venus oriR6K kan)*
LC694	*ΔsoxRS::FRT attλ::(micF'-venus oriR6K kan)*
LC695	*Δrob::FRT attλ::(micF'-venus oriR6K kan)*
LC696	*ΔmarRAB::FRT ΔsoxRS::FRT attλ::(micF'-venus oriR6K kan)*
LC697	*Δrob::FRT ΔsoxRS::FRT attλ::(micF'-venus oriR6K kan)*
LC698	*ΔmarRAB::FRT Δrob::FRT attλ::(micF'-venus oriR6K kan)*
LC699	*ΔmarRAB::FRT ΔsoxRS::FRT Δrob::FRT attλ::(micF'-venus oriR6K kan)*
LC700	*ΔmarRAB::FRT Δrob::FRT*

Table 2.1 (Continued)

LC730	*soxS::cat*
LC731	*JTG1078 soxS::cat*
LC741	*ΔmarRAB::FRT Δrob::FRT soxS::cat soxR*
LC742	*ΔmarRAB::FRT Δrob::FRT soxS::cat soxR105*
LC756	*ΔmarRAB::FRT Δrob::FRT soxS::cat soxR attλ::(mar'-venus oriR6K kan)*
LC757	*ΔmarRAB::FRT Δrob::FRT soxS::cat soxR attλ::(soxS'-venus oriR6K kan)*
LC758	*ΔmarRAB::FRT Δrob::FRT soxS::cat soxR attλ::(rob'-venus oriR6K kan)*
LC759	*ΔmarRAB::FRT Δrob::FRT soxS::cat soxR attλ::(inaA'-venus oriR6K kan)*
LC760	*ΔmarRAB::FRT Δrob::FRT soxS::cat soxR105 attλ::(mar'-venus oriR6K kan)*
LC761	*ΔmarRAB::FRT Δrob::FRT soxS::cat soxR105 attλ::(soxS'-venus oriR6K kan)*
LC762	*ΔmarRAB::FRT Δrob::FRT soxS::cat soxR105 attλ::(rob'-venus oriR6K kan)*
LC763	*ΔmarRAB::FRT Δrob::FRT soxS::cat soxR105 attλ::(inaA'-venus oriR6K kan)*
LC794	*soxS::cat soxR attλ::(soxS'-venus oriR6K kan)*
LC795	*soxS::cat soxR105 attλ::(soxS'-venus oriR6K kan)*
LC910	*ΔompR::cat*
LC951	*Δrob::FRT soxS::cat soxR105 attλ::(soxS'-venus oriR6K kan)*
LC963	*ΔmarRAB::FRT soxS::cat soxR105 attλ::(soxS'-venus oriR6K kan)*
LC998	*ΔtrpD::FRT*
LC1031	*ΔrpoS::FRT attλ::(mar'-venus oriR6K kan)*
LC1032	*ΔrpoS::FRT attλ::(inaA'-venus oriR6K kan)*
LC1054	*Δrob::FRT ΔtolC::cat*
LC1055	*ΔsoxRS::FRT ΔtolC::cat*
LC1056	*ΔmarRAB::FRT ΔtolC::cat*
LC1057	*LC539 ΔtolC::cat*
LC1080	*ΔmicF::kan*
LC1081	*ΔtolC::FRT ΔmicF::kan*
LC1082	*LC539 ΔmicF::kan*
LC1091	*ΔmarRAB::FRT ΔsoxRS::FRT Δrob::FRT attλ::(mar'-venus oriR6K kan) Δcrp::cat*
LC1095	*Δcrp::cat*
LC1096	*LC539 attλ::(micC'-venus oriR6K kan)*
LC1097	*LC539 attλ::(ompF'-venus oriR6K kan)*
LC1099	*LC539 attλ::(ompF'-'venus(hyb) oriR6K kan)*
LC1106	*attλ::(ompF'-venus oriR6K kan)*
LC1108	*attλ::(ompF'-'venus(hyb) oriR6K kan)*
LC1109	*ΔmicF::FRT*
LC1110	*LC539 ΔmicF::FRT*
LC1117	*ΔtolC::FRT ΔmicF::FRT*
LC1126	*ΔentA::cat*
LC1127	*ΔentF::cat*
LC1128	*ΔentE::cat*
LC1129	*ΔtrpE::cat*
LC1130	*ΔentD::FRT attλ::(mar'-venus oriR6K kan)*
LC1131	*ΔentD::FRT ΔtolC::FRT attλ::(mar'-venus oriR6K kan)*
LC1132	*ΔtrpD::FRT attλ::(mar'-venus oriR6K kan)*
LC1133	*ΔtrpD::FRT ΔtolC::FRT attλ::(mar'-venus oriR6K kan)*
LC1136	*ΔentA::cat attλ::(mar'-venus oriR6K kan)*
LC1137	*ΔentF::cat attλ::(mar'-venus oriR6K kan)*
LC1139	*ΔtrpE::cat attλ::(mar'-venus oriR6K kan)*
LC1142	*ΔtolC::FRT ΔentA::cat attλ::(mar'-venus oriR6K kan)*
LC1143	*ΔtolC::FRT ΔentF::cat attλ::(mar'-venus oriR6K kan)*
LC1145	*ΔtolC::FRT ΔtrpE::cat attλ::(mar'-venus oriR6K kan)*
LC1148	*ΔentA::FRT attλ::(mar'-venus oriR6K kan)*
LC1149	*ΔentF::FRT attλ::(mar'-venus oriR6K kan)*
LC1151	*ΔtrpE::FRT attλ::(mar'-venus oriR6K kan)*
LC1152	*ΔtolC::FRT ΔentA::FRT attλ::(mar'-venus oriR6K kan)*
LC1153	*ΔtolC::FRT ΔentF::FRT attλ::(mar'-venus oriR6K kan)*
LC1155	*ΔtolC::FRT ΔtrpE::FRT attλ::(mar'-venus oriR6K kan)*
LC1160	*ΔmarRAB::FRT ΔsoxRS::FRT Δrob::FRT attλ::(mar'-venus oriR6K kan) Δcrp::FRT*
LC1164	*LC539 ΔtolC::FRT*
LC1165	*ΔmarRAB::FRT ΔtolC::FRT*
LC1166	*ΔsoxRS::FRT ΔtolC::FRT*
LC1167	*Δrob::FRT ΔtolC::FRT*
LC1172	*LC1160/pPROTetE*
LC1173	*LC1160/pPROTetE-crp-1*

Table 2.1 (Continued)

LC1178	*ΔtolC::FRT attλ::(ompF'-'venus(hyb) oriR6K kan)*
LC1179	*ΔtolC::FRT ΔmarRAB::FRT attλ::(ompF'-'venus(hyb) oriR6K kan)*
LC1180	*ΔtolC::FRT ΔsoxRS::FRT attλ::(ompF'-'venus(hyb) oriR6K kan)*
LC1181	*ΔtolC::FRT Δrob::FRT attλ::(ompF'-'venus(hyb) oriR6K kan)*
LC1182	*ΔtolC::FRT ΔmarRAB::FRT ΔsoxRS::FRT Δrob::FRT attλ::(ompF'-'venus(hyb) oriR6K kan)*
LC1183	*ΔtolC::FRT ΔmicF::FRT attλ::(ompF'-'venus(hyb) oriR6K kan)*
LC1184	*ΔtolC::FRT attλ::(ompF'-venus oriR6K kan)*
LC1185	*ΔtolC::FRT ΔmarRAB::FRT attλ::(ompF'-venus oriR6K kan)*
LC1186	*ΔtolC::FRT ΔsoxRS::FRT attλ::(ompF'-venus oriR6K kan)*
LC1187	*ΔtolC::FRT Δrob::FRT attλ::(ompF'-venus oriR6K kan)*
LC1188	*ΔtolC::FRT ΔmarRAB::FRT ΔsoxRS::FRT Δrob::FRT attλ::(ompF'-venus oriR6K kan)*
LC1189	*ΔtolC::FRT ΔmicF::FRT attλ::(ompF'-venus oriR6K kan)*
LC1190	*ΔtolC::FRT ΔmarRAB::FRT attλ::(micF'-venus oriR6K kan)*
LC1191	*ΔtolC::FRT ΔsoxRS::FRT attλ::(micF'-venus oriR6K kan)*
LC1192	*ΔtolC::FRT Δrob::FRT attλ::(micF'-'venus oriR6K kan)*
LC1193	*ΔtolC::FRT ΔmarRAB::FRT ΔsoxRS::FRT Δrob::FRT attλ::(micF'-venus oriR6K kan)*
LC1194	*ΔtolC::FRT ΔmicF::FRT attλ::(micF'-venus oriR6K kan)*
LC1200	*ΔmarRAB::FRT attλ::(ompF'-'venus(hyb) oriR6K kan)*
LC1201	*ΔsoxRS::FRT attλ::(ompF'-'venus(hyb) oriR6K kan)*
LC1202	*Δrob::FRT attλ::(ompF'-'venus(hyb) oriR6K kan)*
LC1203	*ΔmarRAB::FRT ΔsoxRS::FRT attλ::(ompF'-'venus(hyb) oriR6K kan)*
LC1204	*Δrob::FRT ΔsoxRS::FRT attλ::(ompF'-'venus(hyb) oriR6K kan)*
LC1205	*ΔmarRAB::FRT Δrob::FRT attλ::(ompF'-'venus(hyb) oriR6K kan)*
LC1206	*ΔmicF::FRT attλ::(ompF'-'venus(hyb) oriR6K kan)*
LC1207	*ΔmarRAB::FRT attλ::(ompF'-venus oriR6K kan)*
LC1208	*ΔsoxRS::FRT attλ::(ompF'-venus oriR6K kan)*
LC1209	*Δrob::FRT attλ::(ompF'-venus oriR6K kan)*
LC1210	*ΔmarRAB::FRT ΔsoxRS::FRT attλ::(ompF'-venus oriR6K kan)*
LC1211	*Δrob::FRT ΔsoxRS::FRT attλ::(ompF'-venus oriR6K kan)*
LC1212	*ΔmarRAB::FRT Δrob::FRT attλ::(ompF'-venus oriR6K kan)*
LC1213	*ΔmicF::FRT attλ::(ompF'-venus oriR6K kan)*
LC1214	*ΔmicF::FRT attλ::(micF'-venus oriR6K kan)*
LC1222	*ΔmarRAB::FRT ΔmicF::FRT*
LC1223	*Δrob::FRT ΔmicF::FRT*
LC1224	*ΔmarRAB::FRT Δrob::FRT ΔmicF::FRT*
LC1225	*ΔmarRAB::FRT ΔmicF::FRT attλ::(ompF'-venus oriR6K kan)*
LC1226	*Δrob::FRT ΔmicF::FRT attλ::(ompF'-venus oriR6K kan)*
LC1227	*ΔmarRAB::FRT Δrob::FRT ΔmicF::FRT attλ::(ompF'-venus oriR6K kan)*
LC1228	*ΔmarRAB::FRT ΔmicF::FRT attλ::(ompF'-'venus(hyb) oriR6K kan)*
LC1229	*Δrob::FRT ΔmicF::FRT attλ::(ompF'-'venus(hyb) oriR6K kan)*
LC1230	*ΔmarRAB::FRT Δrob::FRT ΔmicF::FRT attλ::(ompF'-'venus(hyb) oriR6K kan)*
LC1231	*ΔmarRAB::FRT ΔmicF::FRT attλ::(micF'-venus oriR6K kan)*
LC1232	*Δrob::FRT ΔmicF::FRT attλ::(micF'-venus oriR6K kan)*
LC1233	*ΔmarRAB::FRT Δrob::FRT ΔmicF::FRT attλ::(micF'-venus oriR6K kan)*
LC1239	MDG147 *ΔmarRAB::kan*
LC1240	MDG147 *Δrob::cat*
LC1241	MDG147 *ΔmarRAB::kan Δrob::cat*

Table 2.2. Plasmids used in this work.

Name	Relevant characteristics	Reference
pKD46	*bla* P_{BAD} *gam bet exo* pSC101 *ori(ts)*	(Datsenko and Wanner, 2000)
pCP20	*bla cat cI*857 λP_R'-*flp* pSC101 *ori(ts)*	(Datsenko and Wanner, 2000)
pKD3	*bla rgnB* FRT *cat* FRT *oriR6K*	(Datsenko and Wanner, 2000)
pKD4	*bla rgnB* FRT *aph* FRT *oriR6K*	(Datsenko and Wanner, 2000)
pKD13	*bla rgnB* FRT *aph* FRT *oriR6K*	(Datsenko and Wanner, 2000)
pBAD30	*bla araC* P_{araBAD} *ori*M13 p15A *ori*	(Guzman et al., 1995)
pBAD30-marA	pBAD30::*marA*	
pBAD30-soxS	pBAD30::*soxS*	
pBAD30-rob	pBAD30::*rob*	
pPROTet.E133	*cat* $P_{LtetO-1}$'-6xHN-MCS ColE1 *ori*	Clontech
pPROTet.E-crp-1	pPROTet.E133::*crp*	
pGEX-6P-2	*bla lacI*^{q} P_{lac}'-*gst*-MCS pMB1 *ori*	GE Healthcare
pGEX-marR	pGEX-6P-2::*marR*	
pGEX-rob	pGEX-6P-2::*rob*	
pET28a(+)	*kan* P_{T7lac}'-6xHis-MCS *lacI*^{q} pMB1 *ori*	Novagen
pET28a-marA	pET28a(+)::*marA*	

Table 2.2 (Continued)

pVenus	*kan rgnB yfp(Venus)* t0 *ori*R6K	(Saini et al., 2009)
pVenus-mar	pVenus::P_{marRAB}	
pVenus-soxS	pVenus::P_{soxS}	
pVenus-rob	pVenus::P_{rob}	
pVenus-inaA	pVenus::P_{inaA}	
pVenus-micF	pVenus::P_{micF}	
pVenus-ompF	pVenus::P_{ompF}	
pVenus-FY	pVenus::*ompF'-'yfp(hyb)*	

Chapter 3: A feed-forward regulatory network model for the *mar/sox/rob* regulon of *Escherichia coli* K-12

3.1 Introduction

Bacterial resistance to antibiotics has been a continuing clinical and public health problem for decades. Of the observed mechanisms causing antibiotic resistance there are two primary modes: acquired resistance and intrinsic resistance (Alekshun and Levy, 2007, Nikaido, 2009). Acquired resistance is conferred to bacteria through beneficial chromosomal mutations or more commonly the acquisition of genes encoding antibiotic resistance determinants. Typically, these determinants are carried on mobile genetic elements such as plasmids and transposons (Nikaido, 2009, Alekshun and Levy, 2007, Levy and Marshall, 2004). Intrinsic resistance, on the other hand, is a process where bacterial cells can initiate changes in cellular metabolism and physiology to survive exposure to antimicrobial compounds (Alekshun and Levy, 2007, Nikaido, 2009). A defining feature of intrinsic resistance is that the genes required for this phenotype are naturally occurring in the host's genome.

In the enteric bacterium *Escherichia coli*, a number of naturally existing systems have been implicated in facilitating intrinsic resistance to a broad spectrum of antimicrobial compounds. Among these are multidrug efflux pumps, outer-membrane porins, superoxide dismutases, and metabolic enzymes

(Nikaido, 2009, Alekshun and Levy, 2007, De la Cruz and Calva, 2010). Although many of these systems can independently confer resistance, it is more common that they are used in concert to orchestrate a coordinated response. The ability to coordinate genetic regulation of such diverse genetic targets is mediated by three regulators in *E. coli*: MarA, SoxS, and Rob (Miller and Sulavik, 1996, Martin and Rosner, 2002).

MarA, SoxS, and Rob are three homologous, AraC/XylS-like regulatory proteins involved in the regulation of over 60 genes during exposure to antimicrobial compounds (Barbosa and Levy, 2000, Martin and Rosner, 2002, Gallegos et al., 1997). As a result of this homology, these three proteins bind to a similar, degenerate DNA sequence in promoters leading to the activation or repression of associated genetic targets (Martin et al., 1999, Jair et al., 1995, Jair et al., 1996b, Li and Demple, 1994). Numerous genetic and biochemical studies have identified a common set of regulated genes referred to as the *marA/soxS/rob* regulon. Despite the overlapping nature of the *marA/soxS/rob* regulon, MarA, SoxS, and Rob have been observed to differentially regulate activation and repression of promoters (Martin et al., 2000). This is presumably due to differing affinities of each protein for common DNA binding sites.

In addition to the regulation of downstream genetic targets, MarA, SoxS, and Rob are themselves regulated, albeit by dissimilar mechanisms. MarA expression is driven from the tri-cistronic *marRAB* operon that, including MarA,

encodes genes for MarR and MarB (Cohen et al., 1993a). MarR is the repressor of the *marRAB* operon while MarA is an autoactivator (Seoane and Levy, 1995, Ariza et al., 1994, Martin and Rosner, 1995, Martin et al., 1996). MarB is a protein of unknown function. Up-regulation of the *marRAB* operon can be mediated through binding aromatic compounds such as salicylic acid to MarR resulting in decreased binding affinity of MarR for the *marRAB* promoter (Martin and Rosner, 1995, Cohen et al., 1993b, Alekshun and Levy, 1999a, Seoane and Levy, 1995). Recent evidence also suggests that proteins involved in metabolism and DNA replication are able to interact with MarR resulting in *marRAB* activation (Domain et al., 2007, Domain and Levy, 2010). A unique feature of the *marRAB* system is that both an autorepressor and an autoactivator are encoded in the same operon. This is thought to allow rapid and sensitive response to appropriate environmental stimuli (Martin and Rosner, 2004).

Expression of SoxS is regulated by the redox-sensing regulator, SoxR (Wu and Weiss, 1992, Nunoshiba et al., 1992). SoxR is a Fe-S cluster containing transcriptional regulator found throughout many bacterial species (Park et al., 2006, Hidalgo et al., 1995, Hidalgo and Demple, 1994). On oxidation of the Fe-S cluster in the presence of superoxides and redox-cycling compounds such as paraquat, SoxR increases transcription of *soxS* (Hidalgo et al., 1995, Hidalgo and Demple, 1996). Both SoxR and SoxS autorepress their own expression, respectively, affording the *soxRS* system a rapid shut off mechanism after the

presence of oxidative chemical species have diminished (Nunoshiba et al., 1993b, Hidalgo et al., 1998).

While the regulation of MarA and SoxS expression is at the transcriptional level, Rob is constitutively expressed (~10,000 copies per cell) and is activated post-translationally (Skarstad et al., 1993, Rosner et al., 2002). On binding to chemicals such as dipyridyl, deoxycholate, and decanoate to the C-terminal domain, Rob undergoes a conformational change from an inactive to active form (Rosenberg et al., 2003, Rosner et al., 2002, Griffith et al., 2009). In the inactive state Rob has been observed to form localized aggregates in the cytoplasm, and on activation becomes homogenously dispersed in the intracellular space, leading to a proposed 'sequestration-dispersion' model (Griffith et al., 2009, Ali Azam et al., 1999).

Although these systems exhibit divergent self-regulatory mechanisms, MarA, SoxS, and Rob can also transcriptionally regulate one another. MarA and SoxS, in addition to autoregulating their own expression, have been observed to repress transcription of *rob* (Michan et al., 2002, McMurry and Levy, 2010, Schneiders and Levy, 2006). Likewise, SoxS and Rob have been observed to bind and activate the *marRAB* promoter (Miller et al., 1994, Martin and Rosner, 1997, Martin et al., 1996). The result is a complex regulatory network capable of significant cross-regulation as well as downstream regulation.

Extensive, independent examination of these systems and their genetic targets has defined a complex regulatory system capable of responding to a variety of chemical species. However, an integrated model of the interconnected relationship of the *marRAB*, *soxRS*, and *rob* systems and their associated genetic responses has not been well established. Additionally, a number of global regulators have recently been implicated as activators of the *marRAB* system and ultimately MarA expression (Ruiz and Levy, 2010, Martin and Rosner, 1997, Cohen et al., 1993b). The goal of this chapter is to rigorously define the genetic relationship between the *marRAB*, *soxRS*, and *rob* systems to provide a more accurate picture of the physiological magnitude of genetic cross-talk in the *mar/sox/rob* regulon. Likewise, we explore two global regulators, cAMP receptor protein (CRP) and the stationary phase sigma factor (RpoS), as regulators of *marRAB* transcription.

3.2 Results

The *marRAB*, *soxRS*, and *rob* systems form an integrated regulon. A number of studies have previous shown that the *marRAB*, *soxRS*, and *rob* systems are subject to transcriptional cross-talk (Michan et al., 2002, Schneiders and Levy, 2006, Miller et al., 1994, Martin and Rosner, 1997). Specifically, both SoxS and Rob have been shown to activate the *marRAB* promoter, whereas SoxS and MarA have been shown to negatively regulate the *rob* promoter. Taken

together with corresponding biochemical evidence, it has been suggested that these systems are capable of regulating one another forming a fully integrated regulatory circuit (Schneiders and Levy, 2006, Ruiz and Levy, 2010). To verify this assertion, we constructed a genetic background in which we ectopically expressed each of the regulators MarA, SoxS, and Rob from the arabinose-inducible promoter on a plasmid and monitored gene expression using transcriptional fusions of the *marRAB*, *soxS*, and *rob* promoters to the fast-folding *yfp* variant, Venus (Nagai et al., 2002). The genetic background used lacked the regulatory components *marR*, *marA*, *soxS*, and *rob* in addition to harboring a constitutively active SoxR mutant, *soxR105* (Nunoshiba and Demple, 1994). As P_{soxS} promoter activation is dependent on the oxidation of SoxR, use of a constitutively active (or oxidized) mutant allowed the regulatory role of MarA, SoxS, and Rob on *soxS* gene expression to be examined without the use of additional inducing chemicals.

As shown in **Figure 3.1A**, we found that MarA, SoxS, and Rob could regulate the P_{marR}, P_{soxS}, and P_{rob} promoters in a sign-consistent manner. By this, we mean that these three regulators had the same effect on the activity of each individual promoter, albeit with varying intensities. Consistent with previous studies, we found that MarA, SoxS, and Rob are all activators of the P_{marR} promoter (Miller et al., 1994, Martin and Rosner, 1997, Martin et al., 1996). Interestingly, Rob appears to have a greater ability to activate the P_{marR} promoter than either MarA or SoxS (approximately 1.5 fold higher). This is likely due to the

differences in relative stability of the three regulators as well as their relative levels of expression from the plasmid vector (Griffith et al., 2009, Griffith et al., 2004). Likewise, Rob is able to negatively autoregulate its own expression to similar levels as the negative regulation imposed by MarA and SoxS on the P_{rob} promoter. Finally, both MarA and Rob are able to negatively regulate P_{soxS} transcription, although to a lesser degree than SoxS. Based on these results, we are able to propose the following model for the integrated *mar/sox/rob* regulatory network presented in **Figure 3.1B**.

The effects of transcriptional crosstalk on the activation of the mar, sox, and rob systems to their canonical inducers. We next explored the physiological magnitude of crosstalk in the *mar/sox/rob* network. The model presented in **Figure 3.1B** suggests that all three regulators MarA, SoxS and Rob are capable of modulating the activity of each of the respective P_{marR}, P_{soxS}, and P_{rob} promoters. To directly test this model, we measured the transcriptional activity of these promoters in genetic backgrounds missing all but one of the regulatory systems. This allowed for cross-regulatory effects to be quantified for each system where MarA, SoxS, and Rob are expressed at their physiologically relevant levels. Expression of MarA and SoxS, and activation of Rob, was induced by the addition of salicylate, paraquat or decanoate, respectively, to mid-log phase cultures.

Consistent with the proposed model, and previous observations, both SoxS and Rob are capable of activating the P_{marR} promoter under physiological conditions (**Figure 3.2A**). Although SoxS and Rob are both activators of P_{marR}, these data clearly demonstrate that their relative effects are not equivalent. Presumably, this is due to differences in concentration and stability (Griffith et al., 2004, Martin et al., 2008). Previous studies have shown that Rob is present at 10,000 copies per cell whereas SoxS is present at approximately 2,500 molecules per cell (Griffith et al., 2002, Skarstad et al., 1993). Additionally, SoxS is rapidly degraded by Lon protease whereas Rob is protected from degradation by its C-terminal domain in the 'sequestered' state (Griffith et al., 2009, Griffith et al., 2004). Moreover, promoters of the *mar/sox/rob* regulon have been recently shown to respond proportionally to the amount of MarA and SoxS, termed 'commensurate activation' (Martin et al., 2008). Finally, the regulators MarA, SoxS and Rob have been observed to possess differential binding affinities to regulon promoters (Martin et al., 2000). Particularly, *in vitro* evidence suggests that Rob binds to the P_{marR} promoter with higher affinity than MarA or SoxS (Martin et al., 2002). Taken together, we suggest that Rob is a more abundant, stronger transcriptional activator than SoxS at the P_{marR} promoter.

Examination of the effects on the P_{soxS} promoter yielded unexpected results given the proposed model. Under inducing conditions of either MarA expression or Rob activation, we observed no decrease in P_{soxS} transcription (**Figure 3.2B**). As transcription of P_{soxS} was constitutively activated by the

presence of a constitutively active SoxR (*soxR105*), as to alleviate any additional stress imposed by multiple inducing compounds, we conclude that under physiological conditions MarA and Rob do not significantly decrease P_{soxS} activity.

Finally, we found that both MarA and SoxS are both capable of down-regulating P_{rob} activity *in vivo* although to differing degrees (**Figure 3.2C**). Previous biochemical results have demonstrated that MarA is able to bind P_{rob}, although no *in vivo* data has suggested the relevance of this interaction (Schneiders and Levy, 2006, McMurry and Levy, 2010). Here we show that under salicylate inducing conditions, MarA is capable of mildly repressing P_{rob} activity, although we do note these effects are minor. We likewise observe a decrease in P_{rob} activity via SoxS expression under paraquat exposure, consistent with the results of Michán and coworkers (Michan et al., 2002). Taken together, these results indicate that under chemical induction SoxS remains the only significant repressor of P_{rob} activity.

Autoregulation is a feature of P_{marR} and P_{soxS} but not P_{rob} regulation. Based on the model proposed in **Figure 3.1B,** all three of the associated P_{marR}, P_{soxS}, and P_{rob} promoters are subject to autoregulation. To determine the magnitude of these effects we created genetic backgrounds lacking each of the potential cross-regulatory systems, and measured the effects of transcription in the

presence and absence of the potential autoregulator. This approach eliminates any potential changes in transcriptional activity mediated by indirect activation or repression by the other regulatory systems.

In the case of P_{marR}, we observed MarA-dependent positive autoregulation (**Figure 3.3A**) consistent with current regulatory models (Martin et al., 1996, Ruiz and Levy, 2010). However, we are here able to demonstrate that loss of MarA alone is sufficient to cause a decrease (2.7-fold) in P_{marR} activity during salicylate exposure. Previously, Martin and coworkers arrived at this conclusion by monitoring P_{marR} transcription in a genetic background lacking the entire *marRAB* locus when MarA was overexpressed (Martin et al., 1996). In addition, P_{marR} promoter mutants, with deleted MarA binding sites, were generated and shown to be insensitive to MarA overexpression. They also demonstrated MarA can bind and activate P_{marR} *in vitro*. Based on these lines of evidence it was concluded that MarA is a positive autoregulator of P_{marR}. These data, however, were interpreted when the exact nature of MarA, SoxS, and Rob binding and cross-activation were not entirely clear. Particularly, the *in vivo* data were obtained in genetic backgrounds containing functional Rob (Martin et al., 1996). Our results, though complementary, provide clear, genetic evidence of MarA autoregulation in the absence of Rob and SoxS.

Consistent with our proposed model, we observed SoxS autorepression, though these effects appear to be minor (**Figure 3.3B**). We do not that our

minimal growth medium differs from those used in previous studies that employed a rich growth medium. The effects of growth medium on *mar/sox/rob* activation have been noted, and we have observed greater autorepressive effects in rich medium consistent with Nunoshiba and coworkers (Bailey et al., 2006, Nunoshiba et al., 1993b)(Data not shown). Finally, we found that Rob, despite the model prediction, was unable to autoregulate its own expression in the presence or absence of the inducer decanoate. From these results, we conclude that while MarA and SoxS exhibit positive and negative transcriptional autoregulation, respectively, Rob does not.

Optimal downstream response requires an intact *mar/sox/rob* network. We next wanted to determine the contribution of each system in activating downstream genetic targets of the *mar/sox/rob* regulon under conditions of salicylate, paraquat, and decanoate exposure. To determine the magnitude of these interactions, we monitored gene expression from transcriptional fusions to *inaA* (a putative kinase) and *micF* (the small RNA regulator of *ompF* mRNA translation). Both of these genes are well-characterized members of the *mar/sox/rob* regulon and have been observed to be bound by MarA, SoxS and Rob (Ariza et al., 1995, Jair et al., 1996b, Jair et al., 1995, Li and Demple, 1994, Rosner and Slonczewski, 1994). We found that while each system is capable of exacting a downstream response in the presence of a canonical inducer, it is only

in presence of all three systems that optimal levels of activation are achieved (**Figure 3.4**). As other reports have demonstrated the requirement of *marRAB*, *soxRS*, or *rob* for optimal resistance to antimicrobial compounds (Duo et al., 2008, Warner and Levy, 2010, Cohen et al., 1993a, Cohen et al., 1993b, Hachler et al., 1991), we speculate that this loss of maximal activation is the result of disrupted cross-regulatory interactions. The net result being that the systems are unable to coordinate a response of appropriate magnitude.

Rob responds to salicylate by an indirect mechanism. Although several compounds such as decanoate, dipyridyl and deoxycholate have been observed to bind and activate Rob, recent results have suggested that Rob is subject to activation by salicylate (Rosner and Martin, 2009, Rosenberg et al., 2003, Rosner et al., 2002). We found that while MarA and SoxS activated P_{inaA} and P_{micF} in response to salicylate and paraquat, respectively, Rob alone was capable of activating these promoters in the presence of both salicylate and decanoate (**Figure 3.4**).

We next sought to determine if the interaction between Rob and salicylate is due to direct binding of salicylate to Rob. Previous results have shown that the C-terminal domain of Rob is able to bind dipyridyl and deoxycholate (Rosenberg et al., 2003, Rosner et al., 2002). To determine whether salicylate is bound by Rob, we employed isothermal titration calorimetry (ITC) using purified Rob

protein titrated with a salicylate solution. Our results indicate that no significant changes in free energy were detected during the titration experiment indicated that binding between Rob and salicylate did not occur (**Figure 3.5**). We therefore conclude that Rob is activated by salicylate via an indirect mechanism *in vivo*.

cAMP receptor protein (CRP) activates P_{marR} through direct binding. Unlike the P_{rob} and P_{soxS} promoters, P_{marR} is known to be regulated by global transcription factors. In particular, cAMP receptor protein (CRP) has been implicated as an activator of P_{marR} activation. Though two independent studies have observed decreased P_{marR} activity in *crp* null mutants, no evidence has been presented to show whether the interaction with CRP is direct (Ruiz and Levy, 2010, Zheng et al., 2004). Likewise, these results were obtained in the presence of intact *soxRS* and *rob* systems.

To explore the role of CRP as an activator, we first tested the effects of CRP in a strain lacking *marRAB*, *soxRS*, and *rob* to ascertain whether CRP activates P_{marR} directly or whether CRP-dependent activation requires SoxS or Rob. In the absence of *marRAB*, *soxRS*, and *rob* we observed that transcription of P_{marR} is significantly down-regulated (**Figure 3.6A**). Though these results do not conclusively demonstrate CRP as a direct activator, they do demonstrate that the CRP-dependent effects observed previously are independent of SoxS and Rob.

We next wanted to test whether CRP interacts with the P_{marR} promoter region directly. To determine if CRP binds the P_{marR} promoter, we employed an electromobility shift assay using purified CRP and a 150 bp region of the P_{marR} promoter. Purification of CRP was performed using a 6xHN-CRP fusion protein. We found that this construct was able to fully complement the wild-type levels of P_{marR} transcription (**Figure 3.6B**) *in vivo*. As an *in vitro* positive control we observed that 6xHN-CRP was capable of binding to a 186 bp region of the P_{lacZYA} promoter (**Figure 3.6C**). Performing a similar experiment with P_{marR} we observed that 6xHN-CRP was able to bind to P_{marR} (**Figure 3.6C**). Additionally, this interaction is specific as the introduction of a strong, non-labeled competitor resulted in decreased amounts of shifted DNA. We therefore concluded from these results that CRP is a direct activator of the P_{marR} promoter.

CRP is not the *mar*-independent activator of the P_{marR} promoter. A recurring observation regarding the regulation of P_{marR} has been the existence of a *mar/sox/rob*-independent mechanism for transcriptional activation in the presence of inducing compounds such as salicylate (Cohen et al., 1993b, Martin and Rosner, 1997). Extensive transversion analysis of the P_{marR} promoter region has also demonstrated that the P_{marR} region containing the CRP binding site proposed by Zhang and coworkers (**Figure 3.6D**) is not involved in the *mar*-independent activation mechanism (Martin and Rosner, 1997). Surprisingly, this

analysis did not capture the effects we observe in the absence of CRP (**Figure 3.6A**). We therefore wanted to investigate the possibility of CRP as the *mar*-independent mechanism of P_{marR} activation.

As demonstrated in **Figure 3.6A**, we found that exposure of cells lacking *marRAB*, *soxRS*, and *rob* to salicylate results in persistent activation of P_{marR}, despite the primary activating components MarA, SoxS, and Rob not being present. Furthermore, we still observed activation in the absence of CRP. These results concur with those of Martin and Rosner who determined that, in addition to MarA, SoxS and Rob, both Fis and EmrR (a MarR-family repressor capable of complementing MarR at high copy) do not contribute to the *mar*-independent phenotype (Martin and Rosner, 1997). Taken together, we extend this list of regulators not contributing to the *mar*-independent activation pathway to include CRP.

RpoS is not involved in P_{marR} activation. The transversion analysis of P_{marR} performed by Martin and Rosner also indicated that the only region specifically required for *mar/sox/rob*-independent activation was the RNA polymerase binding sites (Martin and Rosner, 1997). These data strongly suggest that components of RNA polymerase may be the unknown regulatory components. Microarray analysis of cells exposed to salicylate during log-phase growth also indicated that a number of genes under the regulation of RpoS (an alternate RNA

polyermase sigma factor) were up-regulated (Pomposiello et al., 2001). Based on this evidence, we looked to examine the role of the general stress response sigma factor, RpoS, in activating the P_{marR} promoter.

Sigma factors are components of bacterial RNA polymerase involved in promoter recognition and binding. Among the seven sigma factors in *E. coli*, RpoS is involved in general stress responses and the transition to the stationary phase of growth (Klauck et al., 2007). To date, no study has conclusively shown the presence of alternate sigma factor binding sites in the P_{marR} promoter. Likewise, the *marRAB* transcript has been determined to only possess a single start site when P_{marR} is activated by salicylate. However, RpoS recognizes many promoter regions in common with the vegetative growth sigma factor (RpoD) leading us to select RpoS as a possible candidate for a stress response sigma factor.

To test whether RpoS is involved in P_{marR} activation we constructed a mutant lacking *rpoS* in an otherwise wild-type genetic background. We then tested P_{marR} promoter activity in these genetic backgrounds in the presence and absence of salicylate (**Figure 3.7**). Our results clearly demonstrate that RpoS has no apparent role in modulating the transcriptional activity of P_{marR} under the conditions we tested. Further, we examined the possibility of RpoS affecting the activation of a downstream regulon promoter P_{inaA} during salicylate exposure. We also found RpoS does not affect downstream activation.

3.3 Discussion

In this chapter, we have investigated the magnitude of interconnectivity in the *mar/sox/rob* regulatory network. Utilizing a genetic approach we have systematically shown that a fully integrated network, while possible, is not realized under physiological conditions. Consistent with existing models, we have reaffirmed that the interactions of SoxS and Rob with the P_{marR} promoter are the most significant (Miller et al., 1994, Martin and Rosner, 1997, White, 2005). Likewise, SoxS is capable of repressing *rob* gene expression by repressing the P_{rob} promoter (Michan et al., 2002). Based on our results, we propose a model where MarA, SoxS, and Rob form a complex feed-forward regulatory network (**Figure 3.8**) that is highly conditional on the composition of the surrounding chemical environment. Namely, the regulatory network is capable of adopting network topologies based on the induction of one or more of the *marRAB*, *soxRS*, and *rob* systems. Finally, we have shown that *marRAB* gene expression is positively regulated by CRP through direct interaction with the P_{marR} promoter.

An interpretation of this model would suggest that depending on the chemical environment, the *mar/sox/rob* regulon will adopt differing downstream expression profiles in addition to altered response dynamics. Based on the regulatory architecture of positive and negative genetic interactions, feed-forward loops have been observed to confer networks different regulatory properties

(Alon, 2007, Shen-Orr et al., 2002). Therefore, we hypothesize that differing chemical environments capable of inducing two or more of the systems will possess different response profiles than one system alone. We draw support from this idea from the interaction between Rob and the *marRAB* system, both inducible by salicylate. Our results show that although Rob does not appear to respond directly to salicylate, it is through the coordinated action of the Rob and *marRAB* response to salicylate that optimal downstream activation is achieved.

Adding to the complex nature of the *mar/sox/rob* network is the capacity for *marRAB* gene expression to be influenced by global regulators such as Fis and CRP. We have demonstrated here that the interaction between CRP and the P_{marR} promoter is direct, and does not require Rob or SoxS. Based on this finding, we speculate the the *marRAB* system serves as a regulatory link for nutritional information to be fed into the *mar/sox/rob* regulon to produce an appropriate downstream response depending on cellular growth conditions. Ruiz and coworkers have recently demonstrated that loss of either CRP or adenylate cyclase (CyaA) both confer a similar downregulated phenotype in *marRAB* gene expression and that addition of cAMP to the growth media can complement the $CyaA^-$ phenotype (Ruiz and Levy, 2010). Our results, however, show that loss of CRP activity also results in reduced *marRAB* gene expression even in CRP inactivating growth conditions. This implies that, though CRP interacts with P_{marR} directly, there may be additional regulatory elements that feed into the P_{marR} promoter. Further analysis of *marRAB* promoter regulation will continue to

increase the repertoire of transcription factors converging to modulate P_{marR} activity.

In light of the recent findings of Ruiz and coworkers, it is clear that the *marRAB* gene expression is influenced by a large number of global factors, among these is CRP (Ruiz and Levy, 2010). Moreover, the P_{marR} promoter is the only of the *mar/sox/rob* regulatory promoters shown to be subject to global regulation. Therefore, an intriguing hypothesis might be that while Rob and *soxRS* respond to specific chemical signals, the *marRAB* system simultaneously integrates chemical information via MarR derepression, Rob activation, and SoxS expression and global regulatory information via CRP and Fis. An outcome of this scheme is that the feed-forward loops generated by the *mar/sox/rob* regulatory network would differentially activate and repress the large, overlapping regulon depending on the chemical environment and nutritional state of the cell. This therefore underpins the importance of understanding the regulation of *marRAB* gene expression and the role the integrated *mar/sox/rob* network plays in mediating resistance to multiple families of antibiotics when induced.

3.4 Figures

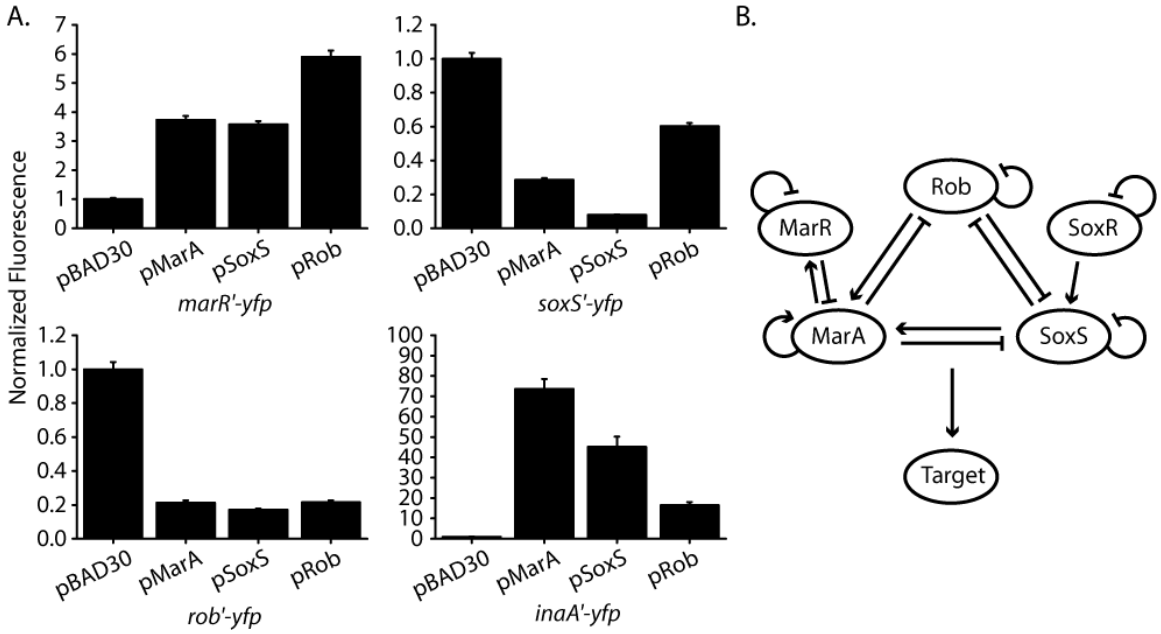

Figure 3.1. The regulatory network formed by MarA, SoxS, and Rob. A.) Determination of MarA, SoxS, and Rob dependent regulatory interactions between the *marRAB*, *soxRS*, and *rob* systems via complementation and monitoring transcriptional activity in a strain lacking all regulatory components except for a constitutively active allele of *soxR* (*ΔmarRAB Δrob ΔsoxS soxR105*). Strains used were LC760, LC761, LC762, and LC763. Cells were transformed with plasmids containing *marA*, *soxS*, or *rob* inserted into the arabinose-inducible pBAD30 expression vector. Transcriptional activity of P_{marR}, P_{soxS}, and P_{rob} were monitored by single-copy transcriptional fusions to *yfp(venus)* inserted at the coliphage λ attachment site. Cells were grown overnight in LB and subcultured 1:200 in fresh LB supplemented with 10 mM arabinose. Following 4 hrs of growth measurements of cellular fluorescence and optical density were made. Measurements were normalized to the empty plasmid control for each of the indicated transcriptional fusions B.) A depiction of the interconnected model as inferred from transcriptional response of P_{marRAB}, P_{soxS}, and P_{rob}.

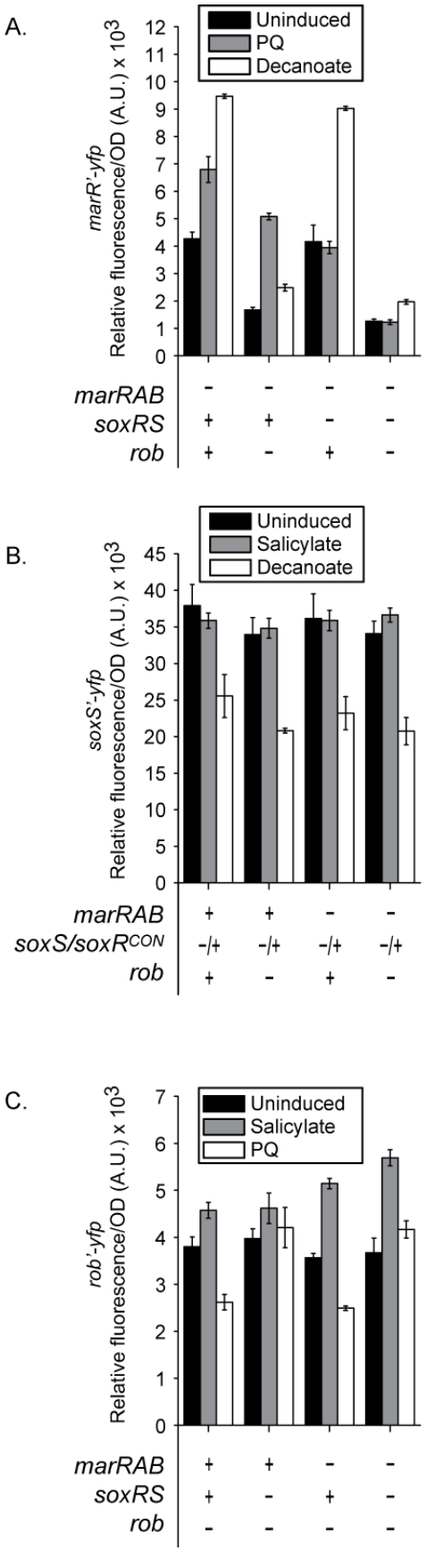

Figure 3.2. The effects of regulatory cross-talk between the *marRAB*, *soxRS*, and *rob* networks. A.) Activity of the P_{marR} promoter by SoxS and Rob (LC665, LC670, LC668, LC671) B.) Activity of the P_{soxS} promoter by MarA and Rob (LC795, LC951, LC963, LC761) C.) Activity of the P_{rob} promoter by MarA and SoxS (LC681, LC683, LC684, LC685). All three regulators are expressed at their native locus. Cells were grown overnight and subcultured 1:200 in MOPS buffered minimal media (20 mM glucose, 0.2% casamino acids, pH=7.2) and grown to an OD=0.5. At this time, canonical inducers salicylate, paraquat (PQ), or decanoate were added to final concentrations of 5 mM, 50 µM, and 5 mM, respectively. Following induction, cultures were grown for additional 1 hr. Fluorescence and optical density measurements were made using 250 µl aliquots of culture in a Tecan Safire2 microplate reader.

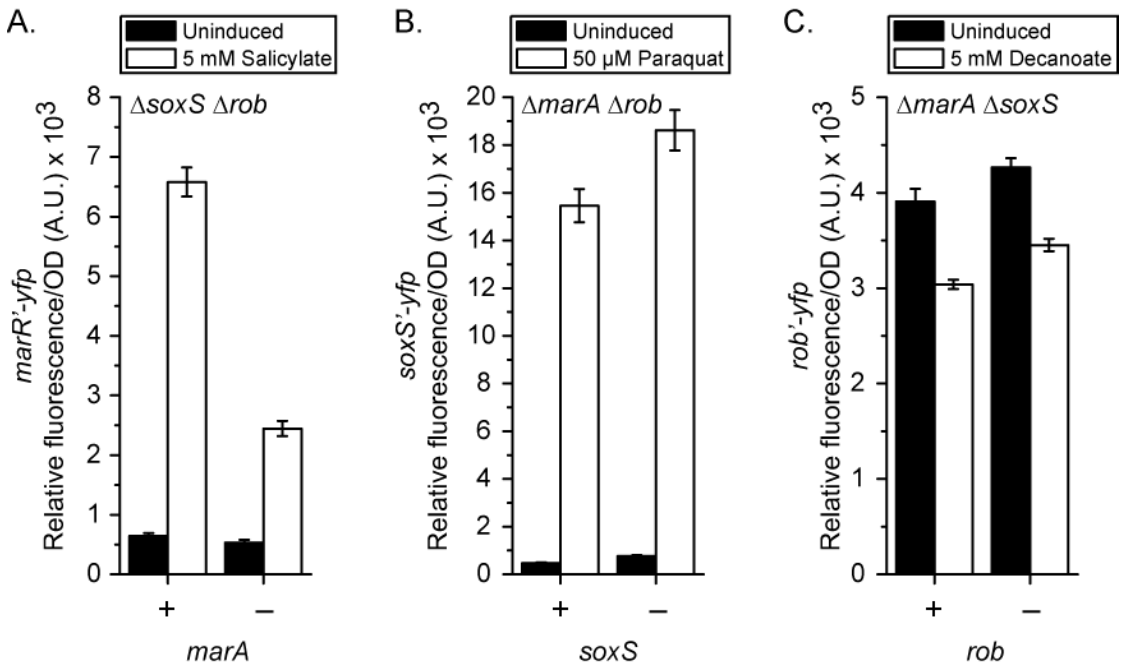

Figure 3.3. Determining the magnitude of autoregulation in the *mar/sox/rob* regulatory circuit. A.) Activity of the P_{marR} promoter in *marA*$^+$ (LC467) and *ΔmarA* (LC487) backgrounds (both *ΔsoxS Δrob*) during salicylate exposure. B.) Activity of the P_{soxS} promoter in *soxS*$^+$ (LC484) and *ΔsoxS* (LC488) genetic backgrounds (both *ΔmarA Δrob*) during paraquat exposure. C.) Activity of the P_{rob} promoter in *rob*$^+$ (LC481) and *Δrob* (LC489) backgrounds during decanoate exposure (both *ΔsoxS Δrob*). Cells were grown overnight and subcultured 1:200 in MOPS buffered minimal media (20 mM glucose, 0.2% casamino acids, pH=7.2) and grown to an OD=0.5. At this time, canonical inducers salicylate, paraquat (PQ), or decanoate were added to final concentrations of 5 mM, 50 μM, and 5 mM, respectively. Following induction, cultures were grown for additional 1 hr. Fluorescence and optical density measurements were made using 250 μl aliquots of culture in a Tecan Safire2 microplate reader.

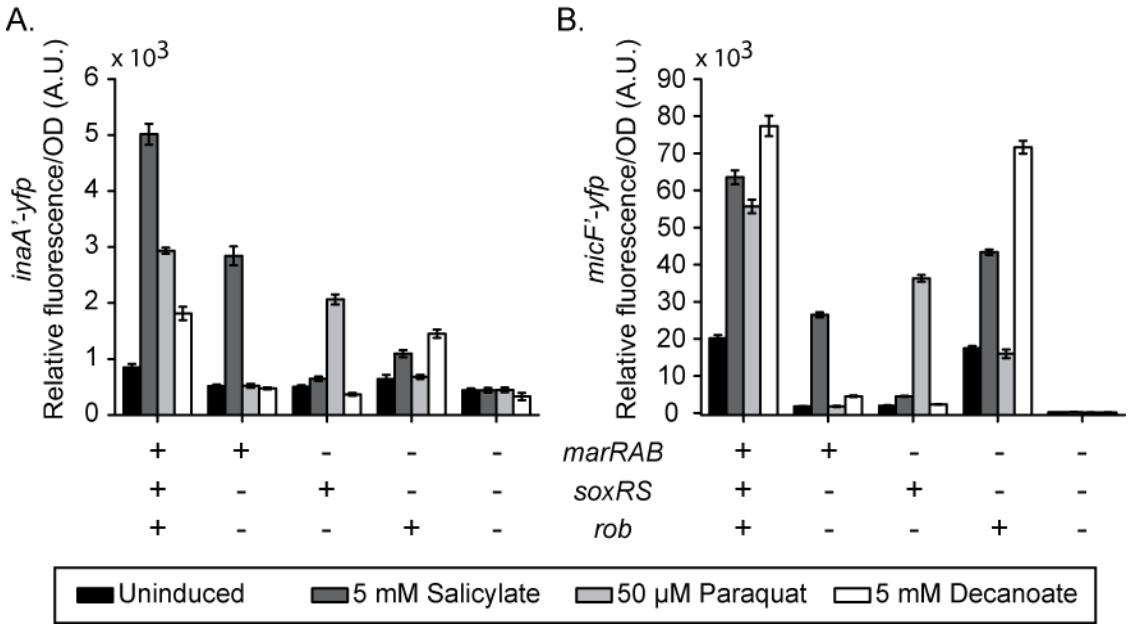

Figure 3.4. The individual contribution of MarA, SoxS and Rob, under native regulation, towards activating two downstream target promoters of the *mar/sox/rob* regulon. A.) Changes in P_{inaA} activation (LC434, LC690, LC691, LC689, LC692) B. Changes in P_{micF} activation (LC439, LC697, LC698, LC696, LC699). Cells were grown overnight and subcultured 1:200 in MOPS buffered minimal media (20 mM glucose, 0.2% casamino acids, pH=7.2) and grown to an OD=0.5. At this time, canonical inducers salicylate, paraquat (PQ), or decanoate were added to final concentrations of 5 mM, 50 µM, and 5 mM, respectively. Following induction, cultures were grown for additional 1 hr. Fluorescence and optical density measurements were made using 250 µl aliquots of culture in a Tecan Safire2 microplate reader.

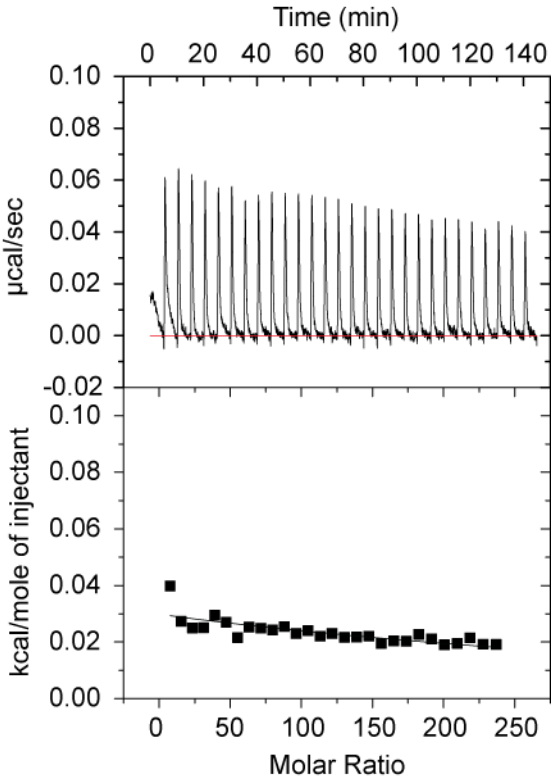

Figure 3.5. Rob does not directly bind salicylate. Measurements were made using a VP-ITC (MicroCal) calorimeter with purified Rob (10 µM) and salicylate (10 mM). Rob was extensively dialyzed against tris-buffered saline (50 mM Tris-HCl, 500 mM NaCl, pH=8.0) and protein concentration was determined by the BCA assay method (Pierce). Sodium salicylate solutions were prepared to 10 mM in tris-buffered saline and the pH was brought to 8.0 by titration with NaOH freshly prior to experiments. Both Rob and salicylate solutions were degassed for at least 15 minutes at room temperature. ITC runs were conducted by titrating 28 x 10 µl salicylate aliquots against the Rob protein sample with constant stirring at 300 rpm and a temperature of 25^{o}C. Data were collected and analyzed using the Origin-based MicroCal software suite.

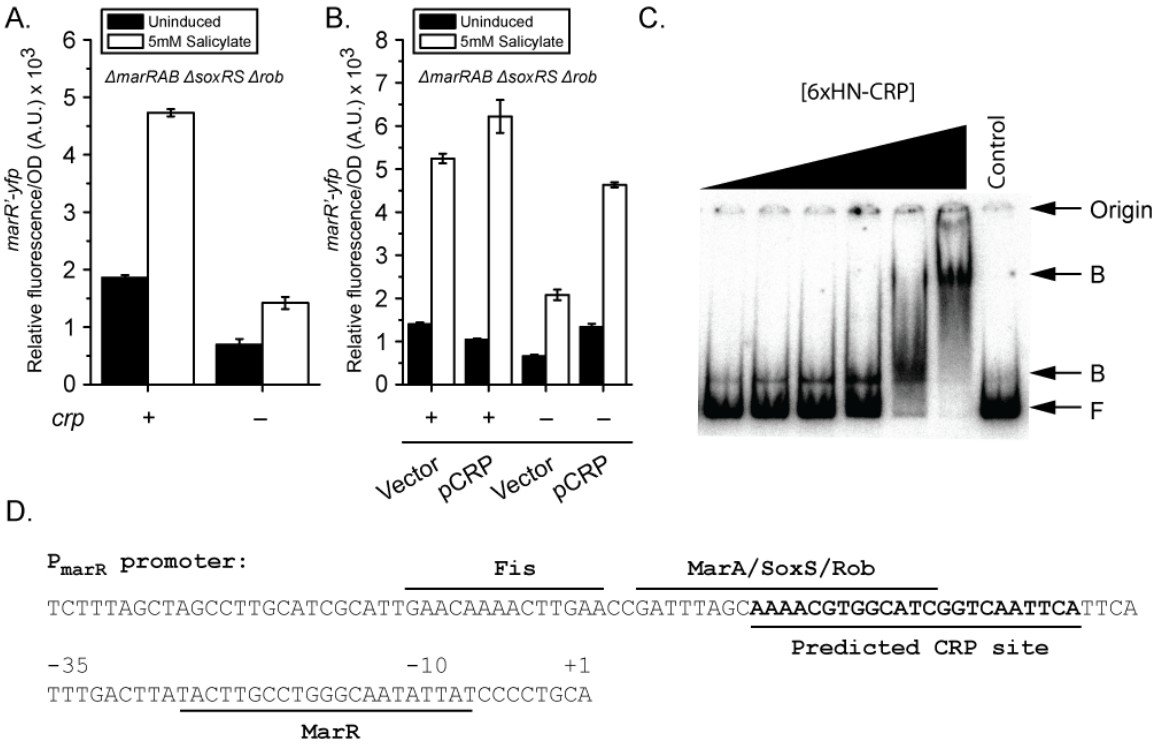

Figure 3.6. Catabolite repressor protein (CRP) activates *marRAB* transcription by binding directly to P_{marR}. A.) Affects of CRP on the background levels of P_{marR} activity in LC671 (*crp*$^+$) and LC1160 (*Δcrp*). Cells were grown in MOPS buffered minimal media (20 mM glucose, 0.2% casamino acids, pH=7.2) B.) Complementation of CRP-dependent activation by 6xHN-CRP expressed from pPROTet.E133 (Vector) and pPROTet.E-crp-1 (pCRP) in LC671 (*crp*$^+$) and LC1160 (*Δcrp*). Cells were grown in MOPS buffered minimal media (20 mM glucose, 0.2% casamino acids, pH=7.2) C. Electrophoretic mobility shift assays of 6xHN-CRP with indicated DNA regions. All binding reactions contained 0.2 mM cyclic-AMP, 10 ng P^{32}-labeled DNA, and 6xHN-CRP (16, 32, 63, 125, 250, 500 ng). Reactions were displayed on a 5% 0.5XTBE polyacrylamide gel containing 0.2 mM cyclic-AMP, run at 150V for 40 minutes at 4^{o}C. D.) The regulatory binding sites present in P_{marR}. The CRP site proposed by Zheng and coworkers (Zheng et al., 2004) is labeled in bold print.

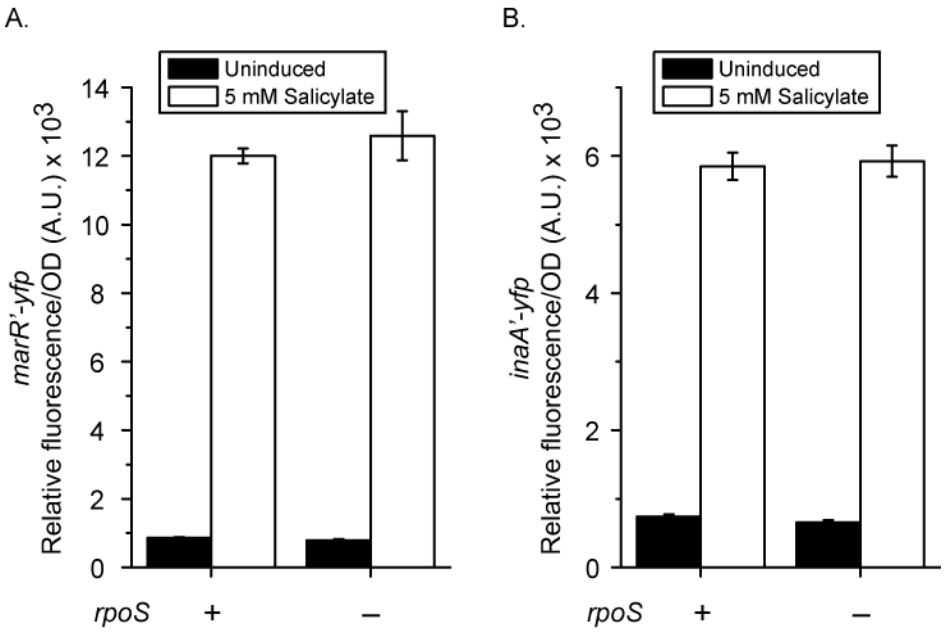

Figure 3.7. RpoS does not contribute to the activation of P_{marR}. Activity of promoters in the presence and absence of salicylate. A.) Activity of the P_{marR} promoter in a *rpoS*$^+$ (LC433) and *ΔrpoS* (LC1031) background. B.)Activity of the P_{inaA} downstream promoter in a *rpoS*$^+$ (LC434) and *ΔrpoS* (LC1032) background. Cells were grown overnight and subcultured 1:200 in MOPS buffered minimal media (20 mM glucose, 0.2% casamino acids, pH=7.2) and grown to an OD=0.5. At this time, salicylate was added to final concentrations of 5 mM. Following induction, cultures were grown for additional 1 hr. Fluorescence and optical density measurements were made using 250 µl aliquots of culture in a Tecan Safire2 microplate reader.

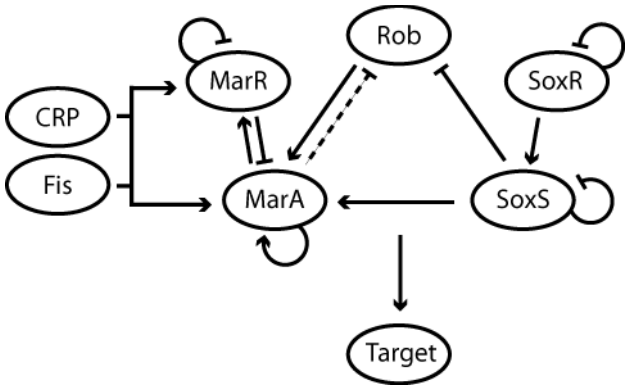

Figure 3.8. A reduced network model of the *mar/sox/rob* network including known global regulators. Dashed lines indicate interactions deemed weak based on the results of this work.

Chapter 4: MarA serves as a conditional autorepressor and set-point controller of its own expression

4.1 Introduction

In the preceding chapter, we have systematically defined the interconnectivity existing in the *mar/sox/rob* regulatory circuits. Of these interactions, we wished to more thoroughly investigate the interactions between Rob and the *marRAB* systems. Particularly, both systems are capable of responding to a common inducer salicylate. For the *marRAB* gene circuit, this response is mediated through the binding of salicylate to the autorepressor MarR, resulting in depression of the P_{marR} promoter and expression of MarA (Martin and Rosner, 1995, Martin and Rosner, 2004, Cohen et al., 1993b). In the case of Rob, however, it is been observed in this work that it does not interact directly with salicylate. We currently hypothesize that Rob is activated by salicylate through a yet unknown, indirect mechanism. Additionally, the *marRAB* circuit possesses a unique regulatory arrangement. The *marRAB* operon contains both positive and negative autoregulators of P_{marR} promoter activation.

Based on the proposed regulatory model in Chapter 3 (**Figure 3.8**), Rob and MarA form a coherent feedforward regulatory loop. A number of experimental and theoretical studies have ascribed specific dynamic properties to

coherent feedforward regulatory networks (Shen-Orr et al., 2002, Alon, 2007). Intrigued by the possibility of an *a priori* predictive model for *mar/sox/rob* regulon activation, we wanted to fully characterize the transcriptional response dynamics associated with the Rob-MarA feedfoward regulatory loop.

In this chapter, we illustrate how MarA positive feedback on the P_{maR} promoter actually serves as a negative feedback, set-point mechanism in the presence of Rob. We propose a model to explain this behavior where MarA is capable of outcompeting Rob for binding at the P_{marR} promoter, but serves as a weaker activator. The net result being that MarA serves to dampen the positive inputs from Rob and additional regulators such as CRP. This in turn produces a fine-tuned response.

4.2 Results

MarA imposes conditional negative feedback on its own expression. A notable feature of the *marRAB* operon is the encoded capacity for MarA-dependent autoactivation (Martin et al., 1996, Cohen et al., 1993a). Previous studies by Martin and coworkers have demonstrated through complementation of MarA, and mutations of the MarA binding box in the P_{marR} promoter, that MarA is capable of positively autoregulating its own expression (Martin et al., 1996). This analysis, as the authors note, was performed in the presence of Rob, although they state Rob was found to not significantly affect their conclusions. Likewise,

loss of *marA* alone was not explored. However, as Rob is a prominent activator of the P_{marR} promoter, we desired to understand the interplay between MarA and Rob in activating P_{marR}.

To quantify the contribution of MarA-mediated autoactivation under physiological conditions, we introduced an in-frame deletion of *marA* into an otherwise wild-type background and in the absence of *rob*. The effects of these deletions on gene activation were then quantified using transcriptional fusions of P_{marR} and P_{rob} to a fast-folding YFP variant, *Venus* (Nagai et al., 2002). Depression of P_{marR} and activation of Rob was mediated with two well-characterized inducers salicylate and decanoate, respectively (Cohen et al., 1993b, Rosenberg et al., 2003, Griffith et al., 2009, Martin and Rosner, 1995).

Despite the apparent autoactivating role of MarA, we observed no loss in P_{marR} promoter activity in the absence of *marA* (**Figure 4.1A**) when activated with salicylate. Interestingly, in the absence of Rob, we found only then did the deletion of *marA* result in decreased levels of P_{marR} activation. An interpretation of these results may be that the binding of Rob at the P_{marR} promoter may block the binding of MarA and thereby inhibiting autoactivation. Alternatively, as MarA has been implicated in the transcriptional repression of *rob* transcription, increased levels of *rob* gene expression in the absence of MarA may account for the epistatic effects of Rob on MarA autoregulation (Schneiders and Levy, 2006, McMurry and Levy, 2010). Although we did observe minor increases in P_{rob}

transcription during salicylate induction (**Figure 4.1B**) in strains lacking *marA*, we suspect based on magnitude of these changes that overall changes in Rob abundance under these conditions remains relatively unchanged.

We next explored the dynamic consequences of P_{marR} activation during salicylate induction. Most strinkingly, we observed that in a *marA* mutant, levels of P_{marR} activation were nearly 35% higher than wild type levels after 2.5 hours of induction (9061±182 RFU/OD vs. 6676±350 RFU/OD, **Figure 4.2A**). Again, in the absence of Rob, we found that P_{marR} activity was decreased. Loss of both *marA* and *rob* ultimately resulted in further inactivation of P_{marR}. We do note the residual activity of P_{marR} in the absence of known activators and is a phenomenon noted in prior studies (Martin and Rosner, 1997). Specifically, treatment of cells with salicylate is known to activate P_{marR} transcription in the absence of all known regulators. Although we have shown cAMP receptor protein (CRP) is a component of this residual activity, additional transcription factors are believed to modulate P_{marR} activity (**Figure 3.6**)(Ruiz and Levy, 2010)

Previous genetic and biochemical analysis has shown that the homologous regulators MarA and Rob each serve as activators of the P_{marR} promoter (Martin and Rosner, 1997). Their role as activators can be demonstrated in the activation of the downstream P_{inaA} promoter (**Figure 4.2B**). Like many of the promoter activated by MarA, SoxS, and Rob, the sites in P_{inaA} are overlapping and are in the forward, class II arrangement (Martin et al., 1999).

Similar arrangements are found in many downstream promoters such as P_{micF}. At the P_{marR} promoter, however, it appears a different mechanism may be at work to produce the increase in activation seen in the absence of *marA*. Based on the structure of the P_{marR} promoter (**Figure 3.6D**), particularly the common binding site for MarA and Rob, we hypothesize that MarA and Rob compete for this site during salicylate induction. Likewise, the overlapping *mar/sox/rob* sites in P_{marR} are in the reverse, class I arrangement suggesting a different binding mode and contacting of RNA polymerase (Martin et al., 1999, Schneiders and Levy, 2006, Schneiders et al., 2004, Martin et al., 2002). Following this logic, we propose a competitive promoter binding and activation mechanism. To attain the observed results in this scenario, given our observations, would be that MarA is able to out-compete Rob at the P_{marR} promoter but is ultimately a weaker activator in comparison to Rob. The net result being MarA, though an activator, is exerting conditional negative feedback on its own expression.

We draw support for this hypothesis from a number of observations. Recent estimates of MarA concentrations place MarA at approximately 9,000 molecules/cell (Martin et al., 2008). Given the levels of Rob range between 5,000 to 10,000 molecules/cell (Skarstad et al., 1993), and Rob is not significantly activated by salicylate (**Figure 4.2D**), we speculate that the concentration of MarA is higher than free Rob under salicylate induction. Likewise, MarA and Rob have relatively similar affinities for the P_{marR} promoter (Kwon et al., 2000). Taken

together, MarA is presumably at higher abundance and therefore able to outcompete Rob for binding.

Modeling results from Wall and coworkers have recently shown that binding of MarA to the P_{marR} promoter does not increase occupancy of RNA polymerase (Wall et al., 2009). Moreover, MarA is able to bind to RNA polymerase very tightly in solution. In competitive binding experiments between MarA, Rob and RNA polymerase, nearly 40-fold higher concentrations of Rob were required to compete with MarA binding to RNA polymerase (Martin et al., 2002). However, this result is complicated by the fact that the *in vitro* behavior of Rob does not accurately reflect the *in vivo*, physiological behavior of Rob (Rosner et al., 2002). Though the exact nature of the competitive interaction is not entirely clear, based on the above evidence, our proposed mechanism explains our data and concurs with prior investigations.

Finally, we explored the range of salicylate dosages where we observed the increase in P_{marR} activation in the absence of *marA* (**Figure 4.2C**). These results indicate that at >1 mM salicylate P_{marR} has elevated levels of activation in a *marA* mutant. Based on this observation we suggest that the effects observed are independent of MarR as it has been shown that extracellular concentrations of 5 mM are required for MarR to be complete unbound from P_{marR} (Martin and Rosner, 1995, Alekshun and Levy, 1999a, Martin and Rosner, 2004). All together, we conclude that MarA outcompetes Rob at the P_{marR} promoter under

salicyate induction, but behaves as a weaker activator. The net result is that MarA conditionally autorepresses its own activation.

MarA and Rob bind P_{marR} with similar affinity *in vitro*. Reports of MarA and Rob affinity for the P_{marR} promoter have varied. Kwon and coworkers previously reported a nearly 10 fold greater affinity for Rob to the *mar/sox/rob* box of P_{marR} compared to MarA (Kwon et al., 2000). Conversely, Martin and coworkers have reported binding coefficients of MarA and Rob to the entire P_{marR} promoter to be much closer in magnitude with Rob binding only marginally stronger. To discriminate between these two results we sought to qualitatively determine if binding of MarA versus Rob was significantly different under equimolar conditions.

Using purified MarA and Rob protein, we performed electromobility shift assays to qualitatively assess differences in DNA binding affinity to the P_{marR} promoter (**Figure4.3**) . We found that despite reports of strongly disparate binding affinities, that MarA and Rob appear to binding with similar affinities. We therefore, based on our qualitative results, support the findings of Martin and coworkers. MarA and Rob bind P_{marR} with similar affinity.

Maximal rate and amplitude of downstream activation requires both MarA and Rob. In addition to affecting the amplitude of genetic responses, feedforward loops have also been observed to alter the rate and timing of genetic responses (Alon, 2007, Saini et al., 2010). As both P_{marR} and Rob are activated by salicyate, and Rob activates P_{marR}, forming a coherent feedfoward loop to activate downstream targets, we wished to explore the impact of MarA and Rob working together to modulate downstream activation. To directly test the effects of MarA and Rob, we monitored gene expression from the P_{inaA} promoter, a well characterized member of the *mar/sox/rob* regulon that responds to both MarA and Rob (**Figure 4.1C**).

The downstream response of P_{inaA}, consistent with our current regulatory model (**Figure 3.8**), shows severe attenuation of P_{inaA} promoter activity in mutants lacking *marA* under salicylate induction (**Figure 4.1C**). Similarly, a *rob* mutation is manifested as the loss of P_{inaA} activity during salicylate and decanoate induction. This is the consequence of P_{marR} promoter activity decreasing in the absence of Rob as well as Rob being activated by both inducing compounds. The net result being that in a *rob* mutant the Rob-MarA feedforward loop is inactivated resulting in a loss in amplitude of downstream regulon activation.

While both the *marA* and *rob* mutants display losses in amplitude of response, their relative effects on response dynamics differ. In the case of a

marA mutant, we observed that activation was delayed and occurred at a much slower rate (**Figure 4.2B**). Conversely, we found that the *rob* mutant exhibited slower rates of gene activation compared to wild type, but faster than the *marA* mutant. From these findings, we conclude that the Rob-MarA feedfoward loop works to increase the rate and amplitude of the *mar/sox/rob* regulon activation during salicylate exposure.

Activation of P_{marR} transcription is a graded response. An interesting response characteristic in genetic regulatory circuits containing positive feedback elements is the emergence of multistability (Guido et al., 2006, Isaacs et al., 2003, Becskei et al., 2001, Maeda and Sano, 2006, Ozbudak et al., 2004, Mitrophanov and Groisman, 2008). If positive feedback is sufficiently strong and cooperative, genetic regulatory networks have been observed to exhibit switch-like activation, wherein a population heterogeneously transitions from a singular off-state to on-state. Conversely, the presence of weak positive feedback or negative feedback has been shown to produce graded or rheostat-like where populations homogenously migrate from an off to on state continuously. An additional physiological aspect of positive feedback is the relative stability of the regulatory elements involved. If the associated regulators are subject to rapid degradation, then it believed the contributions of their positive effects are minimized, resulting in a homogeneous response. As the *marRAB* gene circuit

contains a positive feedback element, we wished to explore the possibility that P_{marR} may act as a dynamic switch as has been observed for other systems (Saini et al., 2010).

To determine which of these cases occurs for P_{marR} activation, we measured fluorescence in single cells using flow cytometry. Populations of induced cells were fixed at 15 minute intervals for one hour of sampling time to monitor the kinetic behavior of the off to on transition in salicylate induced cells. Our results clearly indicate that the P_{marR} has rheostat-like activation profile indicating that MarA-positive feedback is not sufficiently strong enough to induce a bistable switch (**Figure 4.4**). Additionally, MarA is known to be rapidly degraded by the ATP-dependent Lon protease (Griffith et al., 2004). Although this factor was not controlled for in our experiments, we believe this is a contributing factor to the homogeneous activation phenotype although no evidence has been suggested that P_{marR} response dynamics are changed in the absence of Lon protease. Moreover, effects of *lon* mutations are pleiotropic rendering correlation of Lon-dependent effects difficult *in vivo*. These results confirm the assertion of Martin and coworkers who have recently speculated that the *marRAB* circuit would possess a graded response (Martin et al., 2008).

The effects of combined *marRAB* and Rob activation result in additive downstream responses. Several promoters of the *mar/sox/rob* regulon have

been shown to be activated in proportion to the concentration of MarA present (Martin et al., 2008). Likewise, many promoters of the *mar/sox/rob* regulon, including P_{inaA}, have been observed to not reach saturated levels of activation (Martin et al., 2008). This unusual property suggests the potential for synergistic response. As the Rob-MarA feedfoward loop is capable of responding to multiple chemical inputs, we wished to explore the possibility of synergistic regulon activation in the presence of multiple inducers.

To test the possibility of synergistic activation of the Rob-MarA feedfoward loop, we measured promoter activities of the P_{marR} and P_{inaA} promoter after co-induction with variable concentrations of salicylate and decanoate. Based on our observations, found that synergistic activation was not observed (**Figure 4.5**). Specifically, nothing beyond additive levels of activation of P_{inaA} were seen. An interpretation of these results would suggest that while Rob-MarA feedforward loop is important for a strong, timely transcriptional response from the *mar/sox/rob* regulon, it is not capable of producing synergistic transcriptional responses.

4.3 Discussion

In this chapter, we have explored the role of MarA-dependent autoregulation of the P_{marR} promoter. Contrary to the current model of P_{marR} regulation where MarA is an autoactivator, our results indicate that MarA behaves as a conditional

autorepressor of *marRAB* activation (Martin et al., 1996). Additionally, the conditional autorepressive action of MarA is only observed in the presence of Rob. Based on these findings, we propose a competitive binding and activation model to account for the observed transcriptional phenotype of P_{marR} (**Figure 4.6**).

To adequately account for the behavior seen for the levels of P_{marR} activation in a *marA* mutant, we hypothesize that MarA is capable of outcompeting Rob for binding at the P_{marR} promoter. Likewise, of the two regulators, Rob is a stronger activator of P_{marR} promoter activity. In support of this view, we have shown that MarA is capable of competing with Rob at the P_{marR} promoter *in vitro* (**Figure 4.3**). Similarly, our previous results have indicated Rob is a stronger activator of P_{marR} activation than MarA (Chapter 3).

A simple outcome of this proposed model is that MarA conditional autorepression works as a set-point mechanism to ensure appropriate amounts of MarA are produced. Most regulators known to interact with the P_{marR} promoter serve as activators (Fis, CRP, Rob, and SoxS). The result is that, during P_{marR} activation, an array of environmental inputs is capable of increasing MarA expression (**Figure 4.7**). As a result, under certain conditions (namely nutrient limited antibiotic stress), MarA may be expressed at very high levels. Several reports have indicated that overexpression of MarA is detrimental to cellular viability (Griffith et al., 2004). Therefore, we suggest that the autorepressive

nature of MarA at the P_{marR} promoter may serve to balance the net benefit of expressing MarA with the negative outcome of expressing MarA at too high a level.

Additionally, we have shown in this work that the coherent feedforward loop formed by Rob and MarA works to speed up and amplify the downstream transcriptional response of the *mar/sox/rob* regulon. We have also demonstrated that the Rob-MarA feedforward loop acts additively in activating downstream targets. As MarA is capable of autorepressing its own activation, we found this result in accordance with the proposed regulatory model.

Finally, we have demonstrated that activation of the P_{marR} promoter is a graded response. Systems which possess positive feedback often display bistable, switch-like reponses (Mitrophanov and Groisman, 2008). As the *marRAB* circuit contains a positive feedback element, we wished to determine whether a heterogeneous response from this system was possible. Based on our findings, we speculate that the positive feedback imposed by MarA is not sufficiently strong to result in swich-like activation of P_{marR}. Furthermore, given the conditional autorepressive function of MarA, we suspect that the likelihood of a bistable response in the *marRAB* circuit is unlikely.

Of the members of the *mar/sox/rob* regulatory circuits, the *marRAB* system is arguably the most complex. As it is influenced by CRP and Fis as well as MarA, SoxS, and Rob the *marRAB* acts as a regulatory hub in this network

integrating both local and global chemical information (Ruiz and Levy, 2010). Adding to the complex nature of P_{marR} regulation is the conditional autorepressive role of MarA, described in this work. Though the MarA is one of the most extensively characterized transcriptional regulators, the biochemical mechanism at work at the P_{marR} promoter requires further investigation.

4.4 Figures

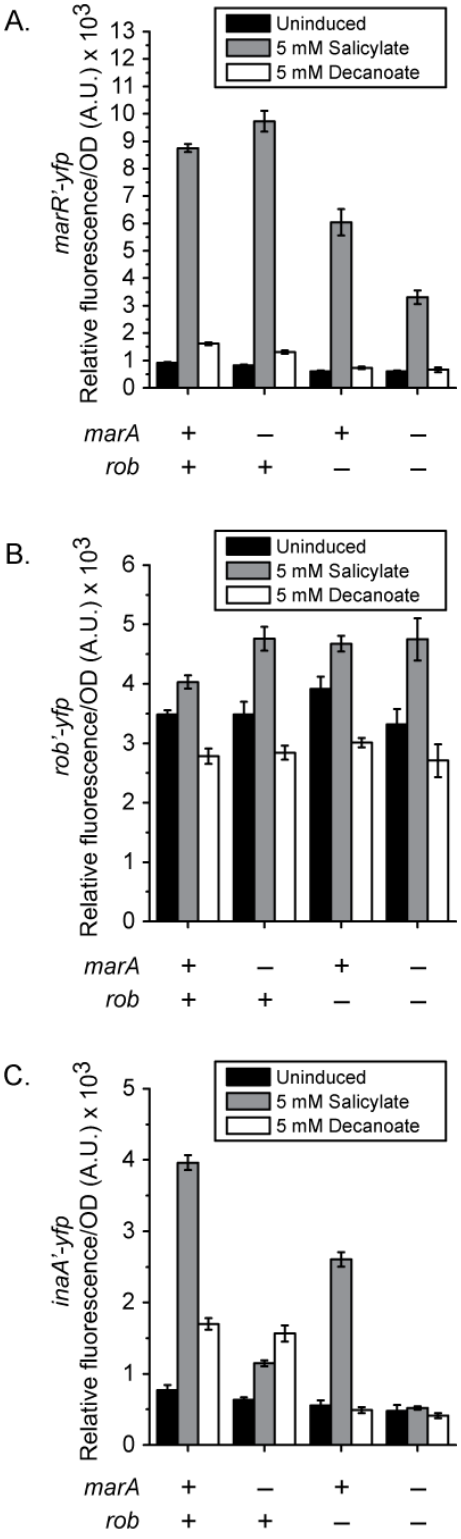

Figure 4.1. Expression of the A.) P_{marR} (LC433, LC475, LC483, LC492) and B.) P_{rob} (LC440,LC477, LC485, LC494) C.) P_{inaA} (LC434, LC478, LC486, LC495) promoters under salicylate and decanoate induction in the presence and absence of *marA* and *rob*. Cells were grown overnight in MOPS buffered minimal medium (20 mM glucose, 0.2% CAA, pH=7.2) and subculured 1:200 fresh medium. Cultures were grown to mid-logarithmic phase (OD=0.5) and induced with 100 µl of media containing concentrated inducer. Final inducer concentration are indicated. Induced cultures were propagated for an additional 1 hr prior to fluorescence and optical density measurements.

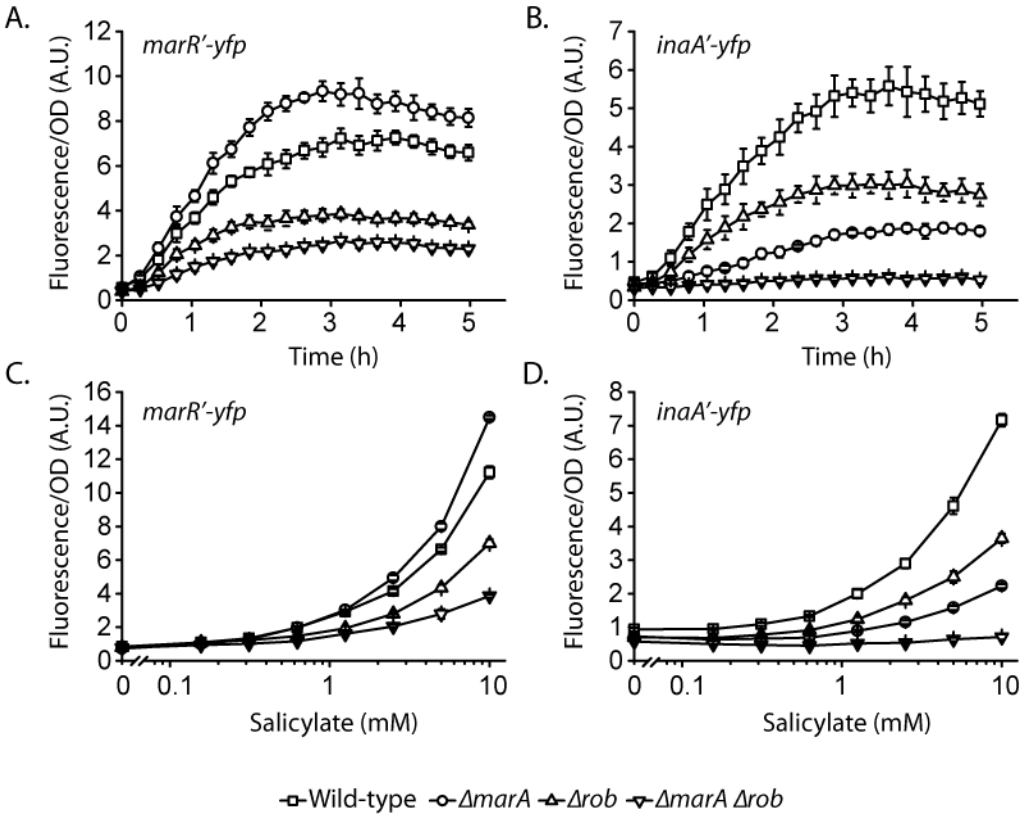

Figure 4.2. Kinetic and dose responses of the P_{marR} and downstream P_{inaA} promoters in response to salicylate. A and B.) Time course gene expression as measured by fluorescence in the presence of 5 mM salicylate. C and D.) Dose-dependent transcriptional response as measured by fluorescence in the presence of indicated salicylate concentrations. All experiments were conducted in MOPS buffered minimal medium (20 mM glucose, 0.2% casamino acids, pH=7.2). For kinetic experiments, overnight cultures were diluted 1:200 and grown for two hours (OD=0.15) prior to induction. Following induction, 150 µl culture was transferred to sterile 96-well microtiter plates and sealed with Breathe-Easy membranes. Plates were incubated in a Tecan Safire2 microplate reader at 37^{o}C with shaking. Fluorescence and optical density measurements were made every 15 minutes as programmed by the instrument. Fluoresence/OD units are reported as x10^{-3}. For end-point measurements, cultures were inoculated as for kinetic experiments, however, induction cultures were grown for 3 hrs prior to measurement (250 µl aliquots).

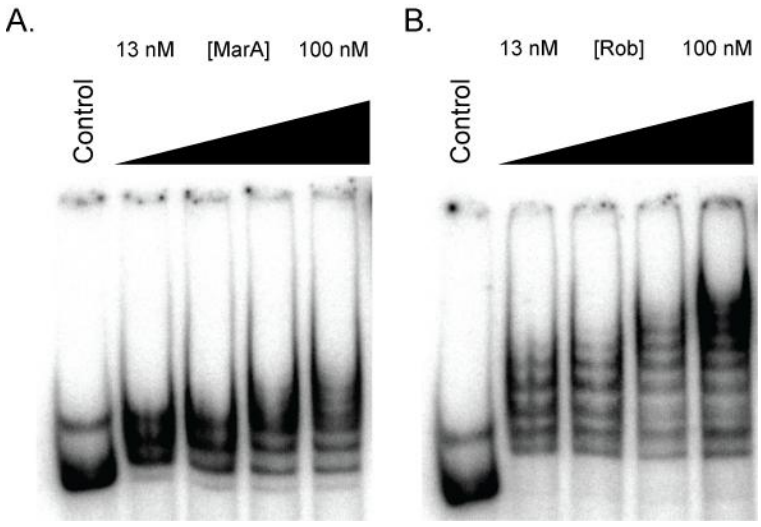

Figure 4.3 MarA and Rob binding to the P_{marR} promoter region. MarA and Rob were supplied at increasing concentration to 2.5 ng of P^{32}-labeled P_{marR} DNA (150 bp fragment used in Chapter 3). Concentrations of MarA and Rob were equimolar (13 nM, 25 nM, 50 nM, 100 nM). Binding reactions were displatyed on 5% acrylamide gels buffered with 0.5X TBE at 150V for 45 minutes. Reactions were displayed on gels run simultaneously. The reaction buffer contained 1 mM $MgCl_2$ in addition to the recipe provided in Materials and Methods.

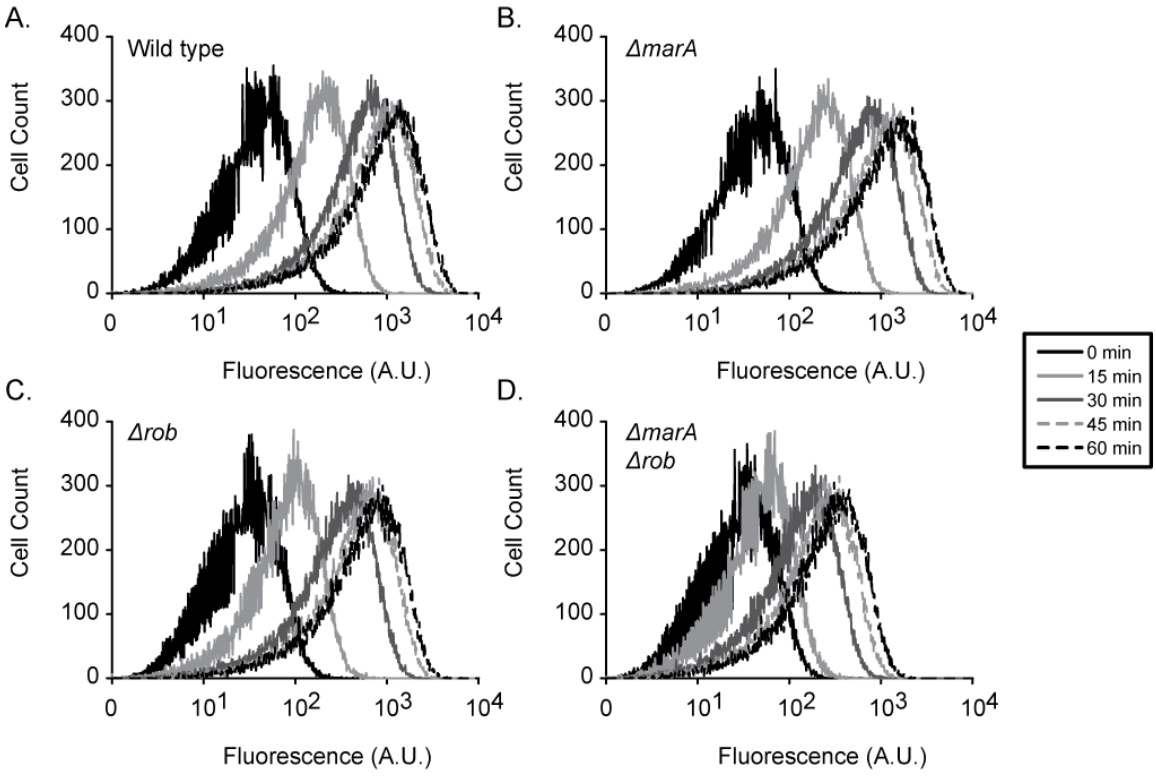

Figure 4.4. Dynamic single-cell response of P_{marR} activation in the presence of 5 mM salicylate as measured by flow-cytometry. Measurements were made from cells fixed every 15 minutes for 1 hour. A.) Wild-type. B.) *ΔmarA* C.) *Δrob* D.) *ΔmarA Δrob*. Cells were grown in MOPS buffered minimal media (20 mM glucose, 0.2% casamino acids, pH=7.2). Subculture (1:200) cells were grown to mid-log phase (OD=0.5) and induced with 5 mM salicylate.

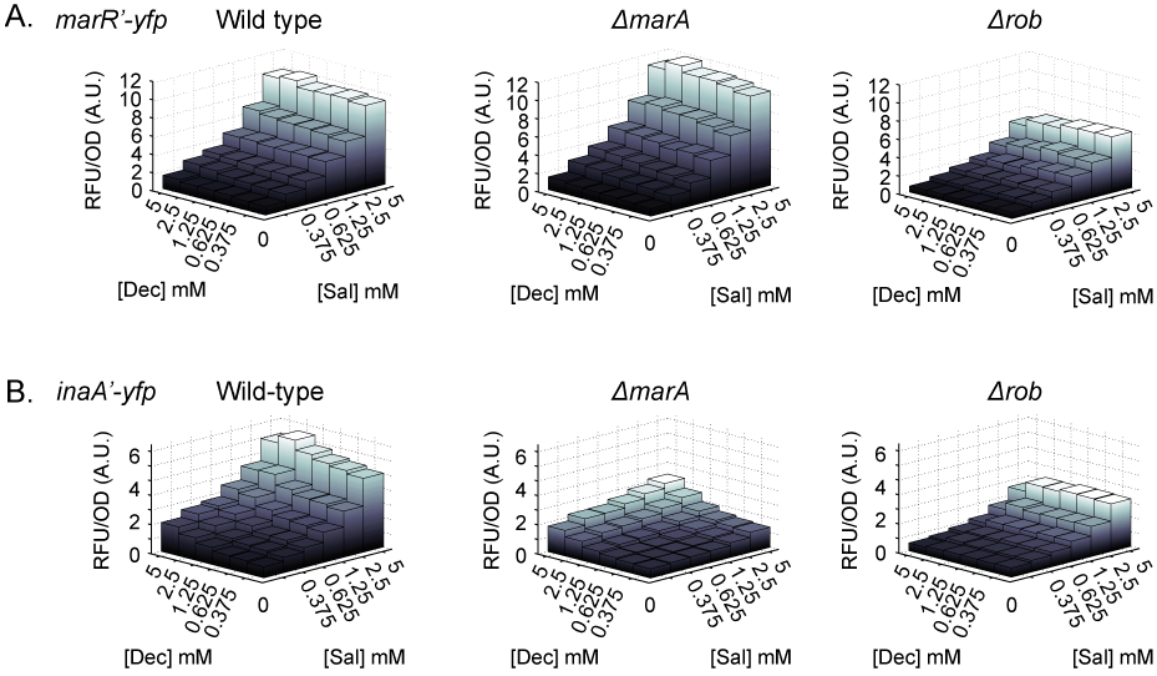

Figure 4.5. Coinduction of cells with salicylate and decanoate. Cells were grown overnight in MOPS buffered minimal medium (20 mM glucose, 0.2% CAA, pH=7.2) and subculured 1:200 fresh medium. Cultures were grown to mid-logarithmic phase (OD=0.5) and induced with 100 µl of media containing concentrated inducer. Final inducer concentration are indicated. Induced cultures were propagated for an additional 1 hr prior to fluorescence and optical density measurements. RFU refers to relative fluorescence. Units reported are $x10^3$.

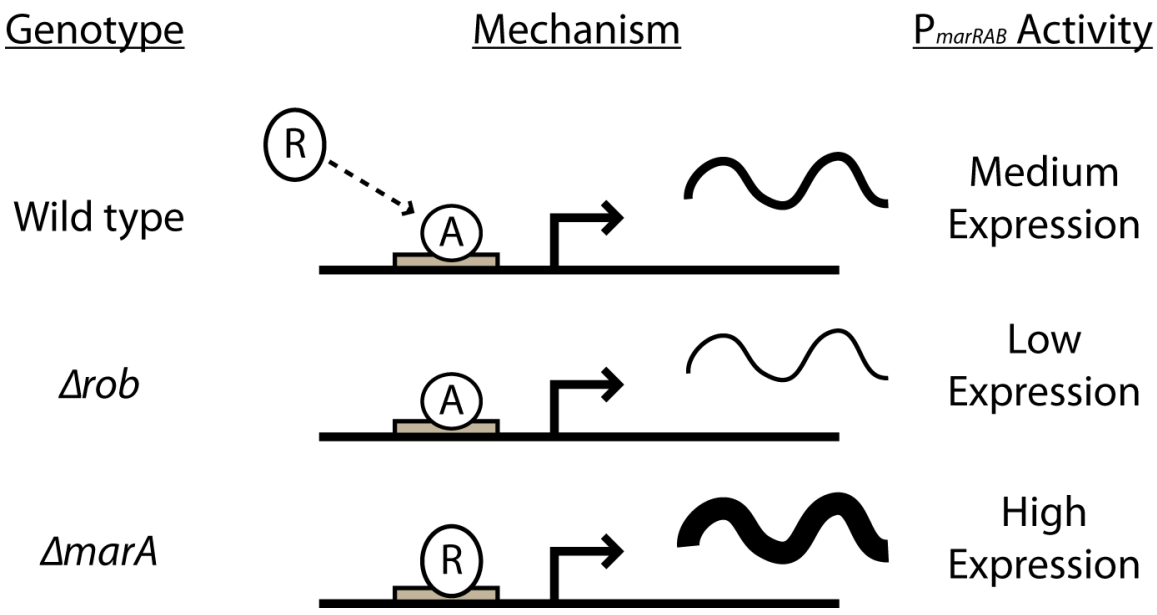

Figure 4.6. Illustration of the competitive binding and activation model proposed for MarA and Rob. A is representative of MarA, R is representative of Rob. Relevant mutant genotypes are indicated.

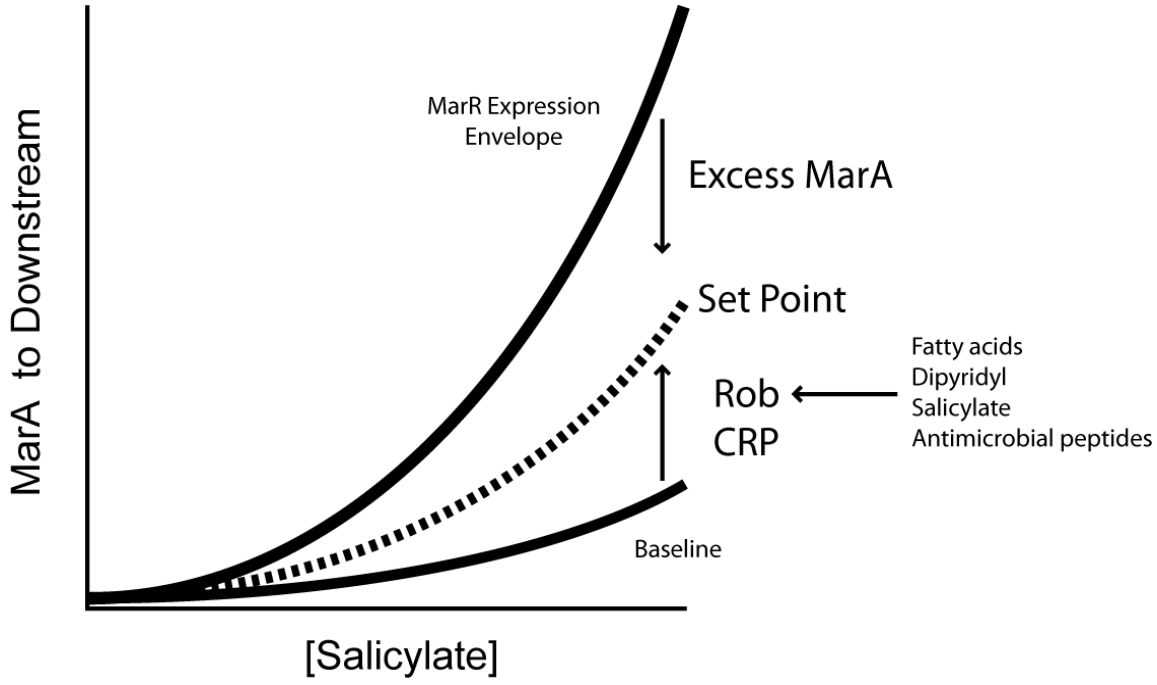

Figure 4.7. A illustration of MarA autoregulatory set-point control.

Chapter 5: The role of MarA and Rob in coordinately modulating OmpF expression at the transcriptional and post-transcriptional levels

5.1 Introduction

One of the initial survival mechanisms for gram-negative bacteria during antibiotic exposure is the alteration of outer membrane porin composition (Pages et al., 2008). By modulating the ratios of these molecular pores, cells are capable of preventing the uptake of membrane impermeable drugs. In *Escherichia coli*, and closely related organisms, this is primarily mediated through changing the ratio of two major outer membrane pores OmpF and OmpC, the large and small porins, respectively (De la Cruz and Calva, 2010, Pages et al., 2008).

Both OmpF and OmpC exist as trimers of 16 stranded β-barrels forming two structurally similar outer membrane pores, but with differing substrate specificities and diffusion rates (Cowan et al., 1992, Basle et al., 2006). Of the two porins, OmpF allows diffusion of larger molecular species and faster rates of diffusion than OmpC (Cowan et al., 1992). Thus, by altering the ratio of these two pores in the outer membrane, gram-negative bacteria are able to exquisitely control the passive uptake of chemicals from the surrounding environment.

Alteration of the OmpF/OmpC ratio is complexly regulated at the transcriptional and translational levels (De la Cruz and Calva, 2010). At the level of transcription, *ompF* and *ompC* gene expression is principally controlled by the EnvZ-OmpR two-component regulatory system (Slauch and Silhavy, 1989, Slauch et al., 1988). Under conditions of low osmolarity, OmpR is primarily unphosphorylated resulting in low concentrations of OmpR-P_i. At low concentrations, OmpR-P_i preferentially binds to high-affinity sites in the P_{ompF} promoter resulting in high levels of *ompF* transcription. Conversely, under conditions of high osmolarity, OmpR is phosphorylated by the activated EnvZ sensor-kinase, resulting in increased OmpR-P_i concentrations. At high OmpR-P_i centrations, OmpR-P_i binds to low affinity sites in the P_{ompF} and P_{ompC} promoters, favoring reduced transcription of *ompF* and increased expression of *ompC* mRNA (Forst et al., 1989, Pratt et al., 1996, Pratt and Silhavy, 1995). Additionally, numerous other regulatory elements converge at the transcriptional level to aid in the effective switching between OmpF and OmpC expression depending on other environmental conditions such as temperature and the presence of membrane destabilizing agents .

In conjunction with transcriptional regulation of *ompF* gene expression are a number of small regulatory RNA (sRNA) species that have been identified to inhibit the translation of the *ompF* mRNA (De la Cruz and Calva, 2010, Vogel and Papenfort, 2006). These sRNA molecules, when expressed, are known to bind to the 5'-untranslated region (5'-UTR) of the *ompF* mRNAs blocking ribosomal

binding to the mRNA, thereby stopping *ompF* translation. Chief among these is the MicF sRNA, the first sRNA regulator to be discovered (Mizuno et al., 1984).

The expression of MicF sRNA, in addition to OmpR-dependent regulation, is modulated by a large number of global transcription factors such as H-NS, Lrp, and IHF (Deighan et al., 2000, Ferrario et al., 1995, Huang et al., 1990). More importantly for the antibiotic resistance phenotype exhibited by *E. coli*, is the action of three homologous regulators MarA, SoxS, and Rob in upregulating *micF* gene expression (Kwon et al., 2000, Martin and Rosner, 2002, Li and Demple, 1994, Gillette et al., 2000). MarA, SoxS, and Rob are three homologous, AraC/XylS-family transcription factors that are the master regulators of the extensive *mar/sox/rob* regulon involved in intrinsic multidrug resistance in enteric γ-proteobacteria (Martin and Rosner, 2002). The regulation of MarA and SoxS expression is chiefly mediated at the level of transcription by the MarR repressor and SoxR redox-senor/activator, respectively (Cohen et al., 1993a, Wu and Weiss, 1992, Nunoshiba et al., 1992). Conversely, Rob is expressed constitutively and activated at the post-translational level by a 'sequestration-dispersion' mechanism (Griffith et al., 2009). The transition of Rob from the inactive to active state is thought to occur through binding of small molecules to its C-terminal domain (Rosenberg et al., 2003, Rosner et al., 2002, Kwon et al., 2000). Further, these three regulators form a complex network of feed-forward regulatory loops to differential manage the downstream regulon (Chapters 3 and 4). Of the regulatory loops formed, the coherent type 1 loop formed by Rob and

MarA is critical for the effective response of *mar/sox/rob* circuit to aromatic, lipophilic toxins such as salicylate.

A notable anomaly of OmpF/OmpC regulation has been the decrease in abundance of OmpF (and correlated increase of OmpC abundance) on the loss of the outer membrane efflux pore TolC (Morona and Reeves, 1982). Understanding of this phenotype was largely ascribed to the action of MicF by Misra and Reeves, who observed that deletions in the *micF* coding region resulted in suppression of the TolC-dependent OmpF/OmpC phenotype (Misra and Reeves, 1987, Misra and Reeves, 1985). Likewise, it was shown that *micF* transcription was elevated in a *tolC* null mutant (Misra and Reeves, 1987, Rosner and Martin, 2009). Recent observations made by Rosner and Martin have also shown that the levels of *marA* and *soxS* expression as well as Rob activation are elevated approximately two-fold in *tolC* null mutants (Rosner and Martin, 2009). The increase in *mar/sox/rob* regulon activation has been attributed to the elevated, intracellular levels of intermediary metabolites that serve as *mar/sox/rob* inducers. Evidence of this effect has been recently been ascribed, in part, to aromatic metabolites in the production of the iron chelator enterobactin (Chapter 6).

Further connecting the *mar/sox/rob* regulon to control of the OmpF/OmpC ratio, is the observation that exposure of cells to salicylate (a well-charactized inducer of MarA expression) is capable of producing an OmpF/OmpC phenotype

similar to that of a *tolC* mutant. The work of Rosner and coworkers has previously demonstrated that salicylic acid exposure results in elevated levels of MicF and reduced OmpF expression and that this phenotype was at least partially OmpR-dependent (Rosner et al., 1991). Subsequent analysis has shown that MarA, SoxS, and Rob are all capable of positively regulating *micF* gene expression. However, their relative roles in reducing OmpF levels has not been fully explored .

A number of outstanding questions exist regarding the previously mentioned results. First, how does the *mar/sox/rob* regulatory loop contribute to the OmpF/OmpC switch? Previously, Cohen and coworkers have shown that while constitute expression of MarA is capable of producing an $OmpF^-$ phenotype, a *marA* mutant was not sufficient to restore a $OmpF^+$ phenotype in the presence of salicylate (Cohen et al., 1993b, Cohen et al., 1988). Second, what elements of the *mar/sox/rob* contribute significantly to the $OmpF^-$ phenotype observed in *tolC* mutants? Does this phenotype require the action of all three systems? In this chapter we address these two questions. We provide genetic and physiological evidence that salicylate response is through the parallel action of MarA and Rob. Moreover, we demonstrate that MarA and Rob affect *ompF* transcription during salicylate exposure. Finally, we show that the effects observed in *tolC* mutants are primarily due to the action of Rob through activating *micF* transcription.

5.2 Results

The salicylate-induced reduction of OmpF and increase of OmpC requires OmpR. Traditionally, analysis of the genetic regulation of *ompF* and *ompC* expression has been conducted in derivatives of *E. coli* K-12 strain MC4100 (Slauch and Silhavy, 1989, Slauch et al., 1988). This strain bears the *ΔlacU169* deletion that encompasses the *lacZYA* operon in addition to a number of other genes including those of the *betIBA* operon (Peters et al., 2003). The *betIBA* operon encodes the genes to synthesis glycine betaine, an osmoprotectant, from choline (Landfald and Strom, 1986). As our experiments are conducted in derivatives of MG1655, which possesses an intact *betIBA* locus, we wished to first determine if the changes in OmpF/OmpC expression would be observed under salicylate exposure.

To determine the effects of salicylate on OmpF/OmpC expression, we harvested insoluble membrane fractions from cells grown in a rich, low osmolarity medium (Kawaji et al., 1979) in the presence or absence of salicylate (**Figure 5.1**). We were able to observe a marked decrease in the levels of OmpF in the outer membrane. Likewise, we observed a moderate increase in the levels of OmpC. Interestingly, Rosner and coworkers observed a decrease in the levels of OmpC when cells were cultured in low-salt Luria-Bertrani media (Rosner et al.,

1991). We suspect the discrepancy between these findings may be due to the differences in strain backgrounds and growth conditions.

We additionally explored the effects of OmpR on regulating OmpF/OmpC expression under salicylate exposure (**Figure 5.1**). Consistent with current regulatory models, neither OmpF or OmpC are expressed in the absence of OmpR. Moreover, we observe that neither OmpF or OmpC are expressed under salicylate exposure. This indicates that salicylate induced OmpF reduction and OmpC increases require OmpR.

Salicylate induced reduction of OmpF requires either Rob activation or MarA expression. Under conditions of salicylate exposure, we have previously shown that both Rob and MarA are required for an optimal downstream, genetic response. Further, it is through the formation of a coherent, type 1 feedforward regulatory loop between Rob and MarA that a strong, rapid transcriptional response is achieved. In short, downstream genetic targets in the *mar/sox/rob* regulon need both Rob and MarA for full activation and MarA expression strongly depends on Rob activation of the P_{marRAB} promoter.

Based on the results of Cohen and coworkers, salicylate induced OmpF reduction was not observed to be due to the action of MarA alone (Cohen et al., 1993b). Given our current regulatory model, we hypothesized that loss of either MarA or Rob alone may not be sufficient to suppress MicF-dependent reductions

in OmpF given that both regulatory systems respond to salicylate. To explore this possibility, we monitored the expression from single-copy transcriptional fusions of *ompF* and *micF* and a translational fusion of *ompF* to the fast-folding YFP variant *Venus* (Nagai et al., 2002). Expression was measured in a series of genetic backgrounds where the *marRAB*, *soxRS*, and *rob* regulatory components of the *mar/sox/rob* network have been systematically deleted. As an additional control, we tested the expression of these fusions in a strain lacking *micF*.

The results of these experiments demonstrate that both MarA and Rob work in parallel to reduce OmpF expression in the presence of salicylate (**Figure 5.2** and **Figure 5.3**). Specifically, we observed a similar 2.5 fold decrease in *ompF* mRNA translation on the loss of either *marRAB* ($MarA^-$) or *rob* (Rob^-). However, no change was observed in the absence of *soxRS* (**Figure 5.2B**). This was also reflected in the levels of expression from the P_{micF} promoter where we found that 1.4 or 2.1 fold decreases were seen on the loss of *marRAB* or *rob*, respectively (**Figure 5.2C**). Most importantly, we discovered that in mutants lacking both *marRAB* and *rob*, the levels of *ompF* mRNA translation were increased >6 fold with correlated decreases in P_{micF} activity of >26 fold.

We also monitored expression of these fusions in mutants lacking *micF*. Given the current regulatory model, disruptions in *micF* should result in comparable levels of *ompF* translation to those observed in a *marRAB rob* (or *marRAB soxRS rob*) double mutant. Surprisingly, we found that the *marRAB rob*

double mutant exhibited 2 fold higher levels of *ompF* translation than the *micF* mutant. These results demonstrate that while *micF* is a determinant in down-regulating *ompF* translation, MarA and Rob appear to regulate *ompF* translation through a MicF-independent pathway. This conclusion is further supported by the phenotypic observation that OmpF levels are higher in mutants lacking *marRAB* and *rob* than mutants lacking *micF* alone (**Figure 5.3**).

All together, these findings show that either expression of MarA or activation of Rob is sufficient to down-regulate OmpF expression under salicylate exposure. We propose this as an explanation for the findings of Cohen and coworkers (Cohen et al., 1993b). Due to the parallel nature of MarA and Rob-dependent regulation of P_{micF}, loss of only one system would not significantly impair the reduction of OmpF. A final conclusion from these data is that although MicF provides a simple mechanism for OmpF reduction, it is clear that an additional MarA/Rob-dependent mechanism exists. Whether this is through the regulation of another sRNA or the indirect alteration of *ompF* mRNA translation is not clear.

MarA and Rob affect levels of transcription from the P_{ompF} promoter. Through monitoring P_{ompF} promoter activity under salicylate exposure, we found that MarA and Rob additively contribute to the levels of P_{ompF} promoter activity (**Figure 5.2A**). Interestingly, the P_{ompF} expression values obtained in the *marRAB*

rob double mutant were nearly identical to those in the *micF* mutant. We speculate that levels of *micF* production, and thereby the levels of OmpF protein expressed, under salicylate exposure may indirectly feedback on the P_{ompF} promoter. Support for this hypothesis can be derived from the observation that both MarA and Rob upregulate *micF* gene expression and loss of both of these regulators results in an apparent $MicF^-$ phenotype (**Figure 5.2C**). Likewise, no evidence currently exists that MarA, SoxS or Rob regulate the P_{ompF} promoter directly (Pomposiello et al., 2001, Barbosa and Levy, 2000). However, whether MarA and Rob may work as accessory regulators to OmpR at the P_{ompF} promoter remains a possibility.

OmpF reduction during salicylate exposure is mediated through a MicF-dendent and a MicF-independent pathway. A notable result from **Figures 5.2** and **5.3** is that levels of OmpF translation and expression in a *marRAB soxRS rob* triple mutant do not match those observed in a *micF* null mutant. These data suggest the possibility of an additional *mar/sox/rob*-dependent pathway for reducing OmpF translation.

To further explore this possibility, we constructed mutants lacking *marRAB* and *rob* in an otherwise *micF* background. In this way, we can then determine which of the key regulators MarA and/or Rob is responsible for regulating the MicF-independent pathway. From the data present in **Figure 5.4**, we observe in

a *micF* mutant that only MarA-dependent factors contribute to further reduction of OmpF expression. Based on these results, we propose the existence of a MicF-independent, post-translational mechanism for the reduction in OmpF expression during salicylate exposure. As these data strongly demonstrate that the effects are mediated at the level of *ompF* mRNA translation, we further propose that the regulatory element involved may be an additional sRNA regulator of *ompF* mRNA translation or stability. Specifically, this mechanism is under the transcriptional regulation of MarA (**Figure 5.4E**).

Upregulation of P_{ompC} promoter activity is independent of Rob. Our results (**Figure 5.1** and **Figure 5.5**) indicate that P_{ompC} transcription is upregulated in the presence of salicylate. These data concur with observations made in previous studies (Rosner et al., 1991). To gather whether MarA and Rob contributed to this increase in activation, we introduced *marRAB* and *rob* deletions into a strain harboring a single-copy transcriptional fusion of *cfp* to the P_{ompC} promoter after the *ompC* coding region (Batchelor et al., 2005). On exposure to salicylate, we observed that expression from the P_{ompC} promoter increases nearly two-fold, consistent with previous findings (Rosner et al., 1991). With regards to MarA and Rob being an effector of P_{ompC} activity, we observed no significant changes in P_{ompC} transcription in a *marRAB*, *rob,* or *marRAB rob* mutant. Collectively, these results demonstrate that P_{ompC} regulation during salicylate exposure is

independent of MarA and Rob despite the existence of *mar/sox/rob* regulatory sites in the close, divergently oriented P_{micF} promoter.

Rob is a suppressor of OmpF reduction in *tolC* mutants. Decreased levels of OmpF expression in *tolC* mutants was a phenotype discovered by Misra and Reeves to be suppressed by deletions in the *micF* coding region (Misra and Reeves, 1987). However, these results were unable to ascribe the source of P_{micF} upregulation that resulted in reduced levels of OmpF. Recent data from Rosner and Martin suggest that the source of increased P_{micF} activity in *tolC* mutants is the result of upregulation of *marRAB*, *soxRS*, and *rob* (Rosner and Martin, 2009). Based on these findings, we wished to determine which of the *mar/sox/rob* systems contribute to the $OmpF^-$ phenotype observed in a *tolC* mutant. Specifically, we studied the effects of MarA, SoxS, Rob, and MicF on the expression of transcriptional and translational fusions described previously.

In the absence of *tolC* we observed that levels of P_{micF} promoter activity were increased and *ompF* translation was decreased, consistent with previous findings. The result of introducing *marRAB*, *soxRS*, and *rob* deletions into the *tolC* mutant background indicated that Rob is the primary mediator of P_{micF} activation, and thereby inhibition of *ompF* mRNA translation through MicF expression (**Figure 5.6B** and **5.6C**). Examining the OmpF content in the outer membrane of these mutants also supports the previous findings (**Figure 5.7**).

Though Rob activation is shown to be the source of increased MicF expression, and ultimately decreased OmpF expression, these findings do not directly indicate the source of Rob activation. Whether this is caused by increased intracellular metabolites or perturbation of other elements of cellular physiology remains to be seen. Collectively, these data indicate that Rob is the primary regulator involved in increased MicF expression in *tolC* mutants. The role of MarA and SoxS in this instance appears to be minor.

5.3 Discussion

In this chapter, we have answered two outstanding questions regarding the regulatory role of the *mar/sox/rob* network in modulating the expression of outer membrane porins OmpF and OmpC. Specifically, we have demonstrated that MarA and Rob function as parallel regulatory pathways to down-regulate OmpF expression during growth in salicylate. Previous reports have shown that the absence of *marA* alone was insufficient to suppress the $OmpF^-$ phenotype produced by salicylate exposure (Cohen et al., 1993b). This was an unexpected finding as constitutive expression of MarA resulted in a similar phenotype to salicylate induced cells (Cohen et al., 1988). Based on our results, we suggest a model where activation of P_{marRAB} and Rob exist as two functionally redundant pathways for P_{micF} promoter activation, MicF expression, and reduced OmpF expression during exposure to salicylate. A consequence of this model is that on

loss of either *marRAB* or *rob*, only a partial decrease in P_{micF} promoter activity is observed, while the overall expression of OmpF remains low enough to produce an $OmpF^-$ phenotype. This provides a near complete explanation for the observations of Cohen and coworkers (Cohen et al., 1993b, Cohen et al., 1988).

As a corollary to examining the role of MarA and Rob in reducting OmpF expression, we provide strong evidence for the existence of a MarA-dependent, MicF-independent pathway for reduction of *ompF* translation. Based on these findings, we also speculate that this may be the result of an unknown sRNA under the regulation of MarA. Traditional microarray analysis employed to study the *mar/sox/rob* regulon would not have detected increased transcription of unknown RNA reading frames. As a result, pinpointing possible sRNA candidates remains a future challenge.

On one hand, this result was not surprising as the expression of MarA and activation of Rob both occur on salicylate exposure. Given the current regulatory model (Chapters 3 and 4), however, this result is of particular interest. Primarily, this observation uncovers a case where the existence of the Rob-MarA feedfoward loop is not relevant for phenotypic regulation. In other words, it would appear that the structure of *mar/sox/rob* feedfoward network may not only be influenced by the regulatory connections amongst MarA, SoxS, and Rob but also by the affinity of these regulators for certain downstream targets. We draw further support for this hypothesis from the observation that MarA, SoxS and Rob are

known to have differential affinity for downstream target promoters (Martin et al., 2000, Martin et al., 2008). Taking this into account with our current observations provides further evidence to the complexity of the *mar/sox/rob* regulatory network and its regulation.

We have also provided a clear regulatory explanation for the observation of Misra and Reeves regarding OmpF reduction in *tolC* mutants (Misra and Reeves, 1987, Morona and Reeves, 1982). Initially, suppressors of the *tolC*-mutant OmpF phenotype were mapped to deletions in *micF*. Likewise, it was found that an increase in P_{micF} promoter activity was present in *tolC* mutants (Misra and Reeves, 1987). Recently, Rosner and Martin have shed light on aspects of this problem by discovering that the *mar/sox/rob* regulon is partially activated in the absence of *tolC* (Rosner and Martin, 2009). Following this line of evidence, we have show that the primary source of P_{micF} promoter upregulation under these conditions is Rob. The exact source of Rob activation in *tolC* mutants remains unknown and the source of future investigation.

An additional point of interest is regarding the role of MarA and Rob in altering the levels of P_{ompF} promoter activation. Under salicylate exposure and in *tolC* mutants we observed that a *marRAB rob* double mutant resulted in nearly 2 fold reductions in P_{ompF} promoter activity. Similarly, we observed the same decreases in activity for *micF* mutants. As MarA and Rob both regulate the P_{micF} promoter, and in their absence P_{micF} promoter activity is reduced to near

undetectable levels, we suspect that this is due to the loss of *micF* and not MarA and Rob directly. A full explanation for this cannot be provided by our results.

5.4 Figures

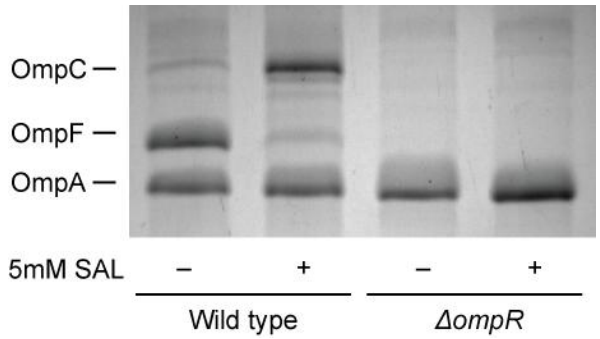

Figure 5.1. The levels of OmpF and OmpC in the outer membrane of MG1655 and a *ΔompR* mutant (LC915). Cells were grown overnight in of Medium A and subculture 1:200 in 10 ml fresh Medium A in absence or presence of 5 mM salicylate. Insoluble membrane fractions were prepared as described in Materials and Methods and displayed on 10% polyacrylamide gel with 6M Urea and SDS.

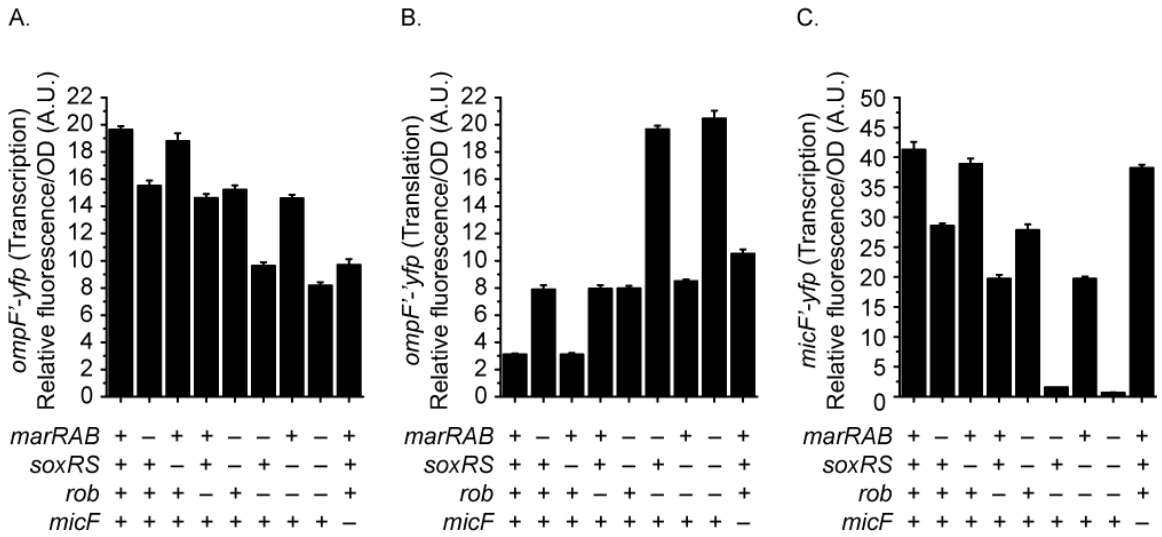

Figure 5.2. Expression from transcriptional and translational fusions in mutants with *marRAB*, *soxRS*, and *rob* systematically deleted. A.) Transcriptional fusion to the P_{ompF} promoter (LC1106, LC1207, LC1208, LC1209, LC1210, LC1211, LC1212, LC1097, LC1213). B.) Translational fusion to the first 13 amino acids of OmpF (LC1108, LC1200, LC1201, LC1202, LC1203, LC1204, LC1205, LC1099, LC1206). C.) Transcriptional fusion to the P_{micF} promoter (LC439, LC693, LC694, LC695, LC696, LC697, LC698, LC699, LC1214). Cells were grown overnight in Medium A and subculture 1:200 in Medium A containing 5 mM salicylate. Cultures were grown in 550 µl aliquots in 2.2 ml, deep well plates with shaking at 1000 rpm for 4 hours. Fluorescence and optical density measurements were made on 250 µl aliquots of culture in a Tecan Safire2 microplate reader.

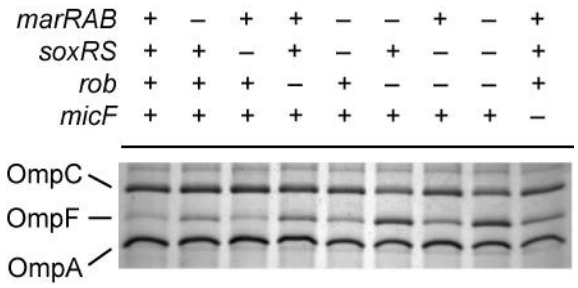

Figure 5.3. The levels of OmpF and OmpC in the outer membrane with systematic deletions in the *mar/sox/rob* regulatory network and *micF* (MG1655, LC496, LC321, LC320, LC497, LC701, LC322, LC539, LC1109). Cells were grown overnight in of Medium A and subculture 1:200 in 10 ml fresh Medium A in the presence of 5 mM salicylate. Insoluble membrane fractions were prepared as described in Materials and Methods and displayed on 10% polyacrylamide gel with 6M Urea/1%SDS.

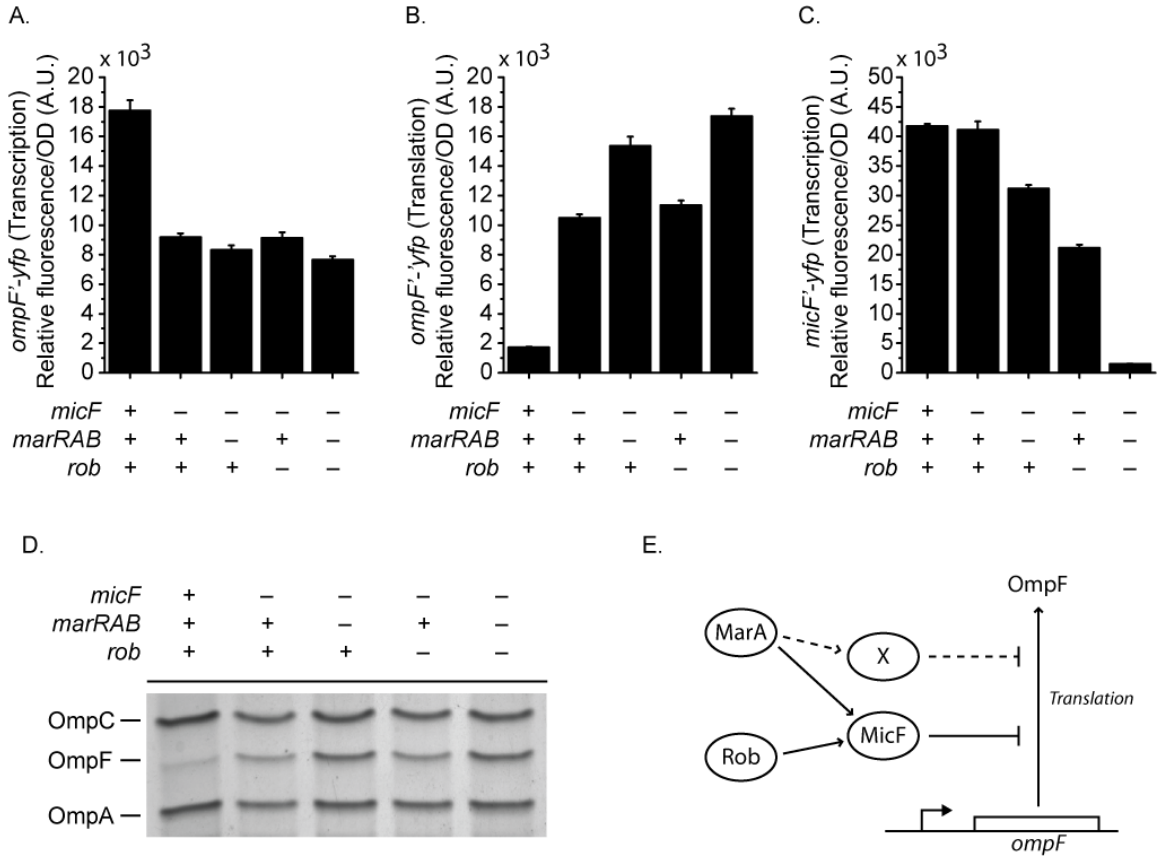

Figure 5.4. A MicF-independent pathway for OmpF reduction is regulated by MarA during salicylate exposure. . A.) Transcriptional fusion to the P_{ompF} promoter (LC1106, LC1213, LC1225, LC1226, LC1227). B.) Translational fusion to the first 13 amino acids of OmpF (LC110, LC1206, LC1228, LC1229, LC1230). C.) Transcriptional fusion to the P_{micF} promoter (LC439, LC1214, LC1231, LC1232, LC1233). D.) Insoluble membrane fractions of mutants grown in 5 mM saliclyate. Cells were grown overnight in of Medium A and subculture 1:200 in 10 ml fresh Medium A containing 5 mM salicylate. Insoluble membrane fractions were prepared as described in Materials and Methods and displayed on 10% polyacrylamide gel with 6M Urea and 1% SDS. E.) A model of the MicF-dependent pathway and its possible regulation.

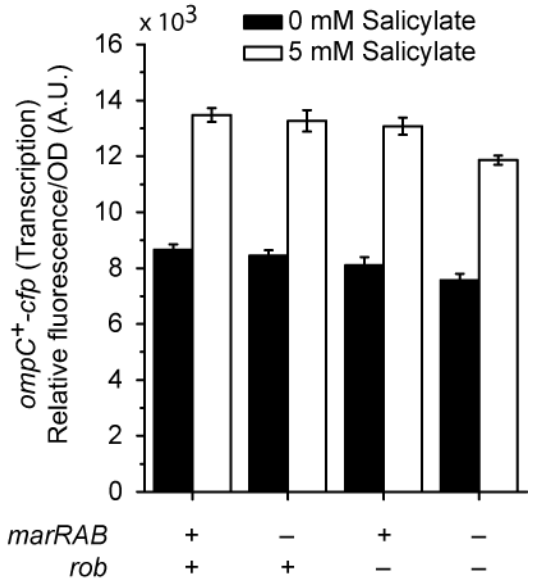

Figure 5.5. MarA and Rob have no apparent affect on *ompC* transcription. Cells were grown overnight in Medium A and subculture 1:200 in Medium A with or without 5 mM salicylate. Cultures were grown in 550 µl aliquots in 2.2 ml, deep well plates with shaking at 1000 rpm for 4 hours. Fluorescence and optical density measurements were made on 250 µl aliquots of culture in a Tecan Safire2 microplate reader.

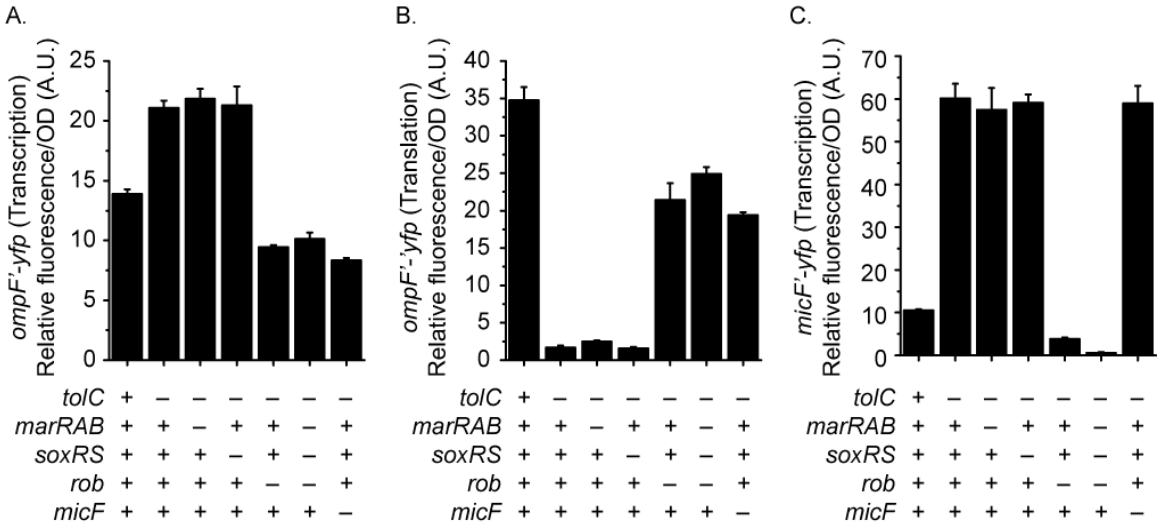

Figure 5.6. Expression from transcriptional and translational fusions in *tolC* mutants lacking *marRAB*, *soxRS*, *rob*, and *micF*. A.) Transcriptional fusion to the P_{ompF} promoter (LC1106, LC1184, LC1185, LC1186, LC1187, LC1188, LC1189). B.) Translational fusion to the first 13 amino acids of OmpF (LC1108, LC1178, LC1179, LC1180, LC1181, LC1182, LC1183). C.) Transcriptional fusion to the P_{micF} promoter (LC439, LC623, LC1190, LC1191, LC1192, LC1193, LC1194). Cells were grown overnight in Medium A and subculture 1:200 in Medium A containing 5 mM salicylate. Cultures were grown in 550 µl aliquots in 2.2 ml, deep well plates with shaking at 1000 rpm for 4 hours. Fluorescence and optical density measurements were made on 250 µl aliquots of culture in a Tecan Safire2 microplate reader.

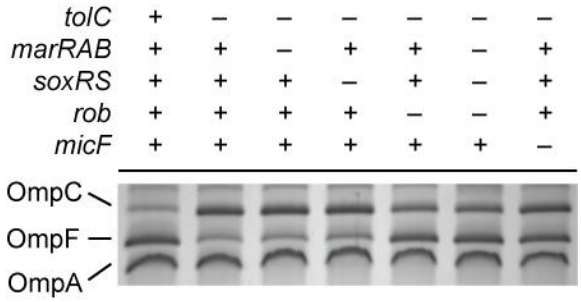

Figure 5.7. The levels of OmpF and OmpC in the outer membrane with deletions in the *mar/sox/rob* regulatory network and *micF* in the absence of *tolC* (MG1655, LC614, LC1165, LC1166, LC1167, LC1164, LC1117). Cells were grown overnight in of Medium A and subculture 1:200 in 10 ml fresh Medium A. Insoluble membrane fractions were prepared as described in Materials and Methods and displayed on 10% polyacrylamide gel with 6M Urea and SDS

Chapter 6: Aromatic acid metabolites of *Escherichia coli* K-12 can induce the *marRAB* operon

6.1 Introduction

The majority of this chapter has been published in the *Journal of Bacteriology* (Chubiz and Rao, 2010). A central regulator of intrinsic antibiotic resistance and organic acid tolerance in *Escherichia coli* and related enteric bacteria is MarR, a negative autoregulator of the *marRAB* operon (Ariza et al., 1994, Cohen et al., 1993a, Cohen et al., 1993c, Maneewannakul and Levy, 1996, Martin et al., 1995, Martin and Rosner, 1995, Alekshun and Levy, 1997, Chollet et al., 2002). This protein is a canonical member of a family of transcriptional repressors commonly associated with regulating the expression of multi-drug efflux systems, stress-response systems, metabolic pathways and virulence factors ((Wilkinson and Grove, 2006) and references therein). A common theme amongst MarR-family regulators is the ability to bind structurally disparate anionic lipophilic compounds. In the case of MarR, numerous chemical compounds such as benzoate, salicylate, 2,4-dinitrophenol, menadione, and plumbagin have been shown to modulate its activity both *in vivo* and *in vitro* (Alekshun and Levy, 1999a, Alekshun et al., 2001, Martin and Rosner, 1995, Seoane and Levy, 1995, Cohen et al., 1993b). Additionally, MarR activity is affected through protein-

protein interactions with enzymes such as transketolase A and DNA gyrase subunit A (Domain et al., 2007, Domain and Levy, 2010).

Recently, Rosner and Martin found that in an *E. coli tolC* mutant the *marRAB* promoter was upregulated approximately two-fold (Rosner and Martin, 2009). They concluded from these results, that upon loss of TolC-dependent excretory capacity, cells accumulate metabolic intermediates capable of inducing the *mar* system. Intrigued by this hypothesis, we examined a number of metabolic pathways and hypothesized that those involved in the superpathway of chorismate may be potential effectors of MarR given their chemical similarity to its known ligands. In this work, we show that three such intermediates in aromatic amino acid biosynthesis activate *marRAB* transcription *in vivo* when supplied endogenously and that one, 2,3-dihydroxybenzoate, directly binds to MarR *in vitro*.

6.2 Results

Aromatic metabolic intermediates activate the *marRAB* operon *in vivo*. Of the several known inducers of *marRAB* expression, the best characterized is salicylate, a weak aromatic acid (Cohen et al., 1993b, Seoane and Levy, 1995, Alekshun and Levy, 1999a, Alekshun et al., 2001, Martin and Rosner, 1995). Specifically, salicylate has been shown to directly bind MarR (Martin and Rosner, 1995, Alekshun et al., 2001). If metabolic intermediates are potential ligands for MarR, we surmised that that they may have similar chemical structure to the

canonical inducer salicylate. Among the vast number of metabolic intermediates in *E. coli* metabolism, those involved in the superpathway of chorismate appeared to be the most promising (Keseler et al., 2009).

Examination of this superpathway in *E. coli* K-12 yielded four compounds that were structurally similar to salicylate (**Figure 6.1A**). Our basis for assessing similarity was the presence of a carboxylate group with adjacent hydroxyl or amine groups on the benzyl ring. Four putative compounds were identified: 4-hydroxybenzoate (HBA), an intermediate in ubiquinone-8 biosynthesis; 2,3-dihydroxybenzoate (DHB), an intermediate in enterobactin biosynthesis; anthranilate (ANT), an intermediate in tryprophan biosynthesis; and 4-aminobenzoate (PABA), an intermediate in tetrahydrofolate biosynthesis.

To test whether these compounds were inducers of the *marRAB* operon, we used a chromosomal, single-copy transcriptional fusion of the *marRAB* promoter to the fast-folding YFP variant *Venus* to monitor gene expression in the presence of the four potential inducers (Nagai et al., 2002). For comparison, we also explored the ability of these chemicals to activate expression in a *tolC* null mutant. Of the four, we found that only DHB and ANT activate the *marRAB* promoters in wild-type cells (**Figure 6.1B**). We also found that HBA could activate the *marRAB* promoter in a *tolC* null mutant. Interestingly, PABA failed to activate the *marRAB* promoter despite its chemical similarity to salicylate.

MarR binds 2,3-dihydroxybenzoate. We next tested whether MarR directly binds to DHB, ANT, and HBA. To measure binding, we employed isothermal titration calorimetry (ITC) using purified MarR (**Figure 6.2**). Consistent with previous measurements, we found that the affinity of MarR for salicylate was K_d=0.9 mM, well within the ranges reported using other methods (Martin and Rosner, 1995, Alekshun and Levy, 1999a). Binding was not observed with isopropyl β-D-1-thiogalactopyranoside (IPTG), which served as our negative control. We also found that MarR bound DHB with a K_d=0.5 mM. However, we found that ANT did not bind to MarR even though it was capable of activating the *marRAB* promoter. Similarly, we found that HBA, which activates the *marRAB* promoter only in the absence of *tolC*, also did not bind to MarR (data not shown). This suggests that these two compounds may indirectly regulate *marRAB* promoter activity.

We note that despite MarR having similar affinities for the two, salicylate has a greater effect than DHB on *marRAB* activation *in vivo*. To explain these results, we imagine that DHB is likely being metabolized by the cell. Consistent with this hypothesis, we observed increased levels of DHB-dependent *marRAB* promoter activation in a *tolC* mutant background (**Figure 6.1B**), suggesting loss of the excretory function of TolC may lead to a buildup of DHB, most likely by preventing the efflux of the downstream metabolite, enterobactin (Bleuel et al., 2005).

MarR activity is modulated *in vitro* by 2,3-dihydroxybenzoate. As MarR binds to DHB, we next tested whether it affects MarR binding to the *marRAB* promoter region. To determine loss of MarR binding activity in the presence of DHB, we employed electrophoretic mobility shift assays using purified MarR and a 150 base-pair region of *marRAB* promoter (see Supplement for details). Using salicylate as our positive control and IPTG as our negative control, we found that only DHB directly affected MarR activity (**Figure 6.3**). Interestingly, we observed significantly more unbound DNA in the presence of DHB than salicylate, even though MarR has similar binding affinities for the two. We also tested whether ANT affected MarR activity and found that it did not, consistent with our ITC experiments.

Disruptions in enterobactin and tryptophan biosynthesis affect *marRAB* promoter activity in *tolC* mutants. Our previous results have demonstrated that DHB and ANT activate the *marRAB* promoter *in vivo* when added exogenously. To better correlate the metabolite effector hypothesis of Rosner and Martin to these observations, we disrupted enzymatic steps in enterobactin and tryptophan metabolism that block either the synthesis or utilization of DHB or ANT. Specifically, mutants lacking the enterobactin synthesis pathway (*ΔentCEBAH*) or deficient in 2,3-dihydro-2,3-dihydroxybenzoate (DDHB) dehydrogenase (*entA*), enterobactin synthase (*entF*), anthranilate synthase (*trpE*), and phoshoribosyl transferase (the C-terminal region of *trpD*) activities were constructed in

otherwise wild type and *tolC* mutant strains and tested for alterations in *marRAB* promoter activity.

In TolC$^+$ backgrounds, we observed no significant changes in *marRAB* promoter activity in any of the mutants, presumably due to the ability of these cells to readily excrete accumulated intermediates (data not shown). However, when we repeated these experiments in the absence of TolC, we found that *marRAB* promoter was less active in mutants unable to synthesize enterobactin from chorismate (*ΔtolC*: 1559±170 vs. *ΔtolC ΔentCEBAH*: 1387±195 RFU/OD, P=0.001). However, when we attempted to accumulate the DHB in the cell by blocking conversion of DHB to enterobactin, we observed a decrease in *marRAB* promoter activity as opposed to an expected increase (*ΔtolC*: 1559±170 RFU/OD vs. *ΔtolC ΔentF*: 1491±78 RFU/OD, P=0.04). While statistically significant, the effect is minor and likely not physiologically significant. We suspect that this mutant (*ΔtolC ΔentF*) does not accumulate significant amounts of DHB. Interestingly, we observed a significant increase in *marRAB* promoter activity when we blocked the conversion of DDHB to DHB (*ΔtolC*: 1559±170 vs. *ΔtolC ΔentA*: 1737±133 RFU/OD, P=0.0001). Given the similar chemical structures of DDHB and DHB, differing only by a hydrated 2,3 carbon-carbon bond on the benzyl ring, we suspect that DDHB may accumulate in this mutant and serve as an alternate activator of MarR. Collectively, these results suggest that enterobactin intermediates are physiological activators of the *marRAB* operon.

We also investigated the effects of tryptophan biosynthesis on *marRAB* expression in the absence of TolC. We found that mutants unable to convert ANT to tryptophan did not exhibit any significant changes in *marRAB* promoter activity (data not shown). Interestingly, in the absence of anthranilate synthase, and therefore the ability to synthesize ANT, we observed a statistically significant increase in the *marRAB* promoter activity (*ΔtolC*: 1516±68 vs. *ΔtolC ΔtrpE*: 1702±173 RFU/OD, $P<0.00002$). We suspect that blocking the initial step of tryptophan biosynthesis may redirect metabolic fluxes to other pathways where the metabolitic intermediates induce *marRAB* expression. Taken together, these results indicate that disrupting tryptophan biosynthesis affects *marRAB* expression, though they suggest that tryptophan intermediates do not contribute directly to the *tolC* phenotype. Moreover, the effect is not direct nor is the mechanism clear. We were not entirely surprised by this result as we found that ANT does not directly bind MarR and affect its activity. Rather, the effect appears to be indirect.

6.3 Discussion

A number of studies have shown that some metabolic intermediates are inducers and substrates for various efflux pumps (Carole et al., 1999, Van Dyk et al., 2004, Helling et al., 2002, Liu et al., 1999a, Liu et al., 1999b). Likely, these mechanisms prevent the buildup of toxic metabolic intermediates. In support of this model, our results demonstrate that MarR directly binds one such

intermediate, 2,3-dihydroxybenzoate, involved in biosynthesis of enterobactin, itself a substrate for TolC (Bleuel et al., 2005). Whether DHB is a MarR effector under physiological conditions, however, is still unknown.

While these results suggest that enterobactin biosynthesis contributes to the increase in *marRAB* expression in the absence of TolC, they do not explain the phenotype completely. Due to the magnitude of the changes in *marRAB* expression caused by mutations in the enterobactin pathway, we suspect that the true source of increased activation is likely a combination of many intracellular metabolites as proposed by Rosner and Martin (Rosner and Martin, 2009).

6.4 Figures

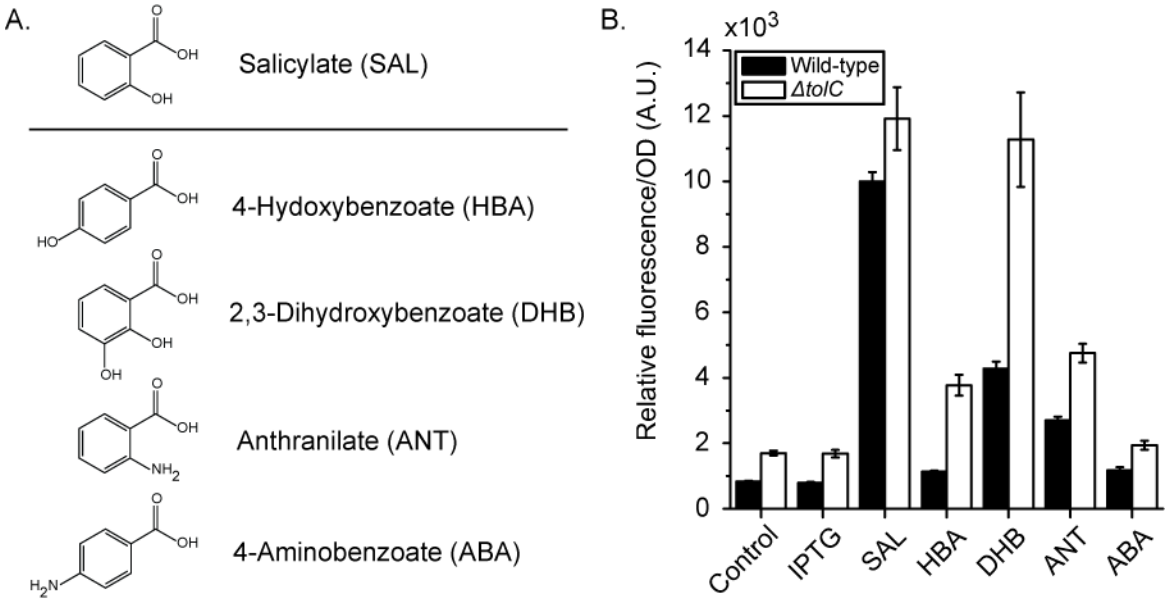

Figure 6.1. Activation of the *marRAB* operon by aromatic acid metabolites in the superpathway of chorismate. (A) Chemical structure of metabolites selected based on their similarity to salicylate. (B) Observed transcriptional activation of the *marR'-yfp* promoter fusion in the presence of 5 mM concentrations of indicated inducers in wild-type (LC433) and *ΔtolC* mutant (LC621) backgrounds. Salicylate (SAL) and IPTG served as positive and negative controls for activation, respectively. Cells were grown overnight in MOPS minimal media (20 mM glucose, 0.2% casamino acids, pH = 7.2) and subcultured 1:200 in fresh media. Following dilution, 450 µl of culture was transferred to deep 96-well plates and grown at 37^{o}C with aeration at 1000 rpm to an OD=0.5. At this time, 100 µl of media containing dissolved inducer was added. Growth was continued for an additional 2 hours prior to fluorescence and optical density measurements made with a Tecan Safire2 microplate reader.

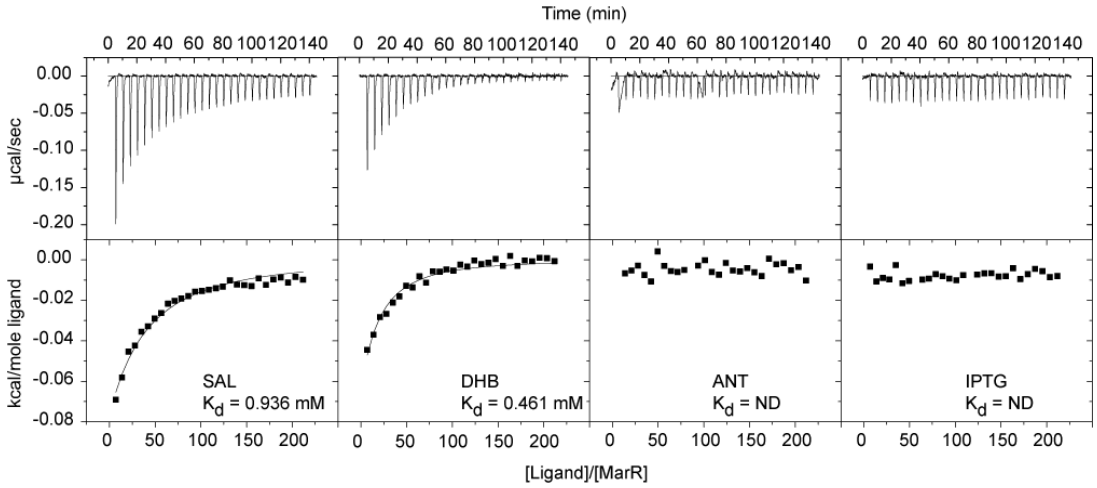

Figure 6.2. MarR binding of aromatic acid inducers as determined by isothermal titration calorimetry (ITC). Experiments were conducted using a VP-ITC calorimeter (MicroCal) with 1.4 mL MarR at 10 µM and ligands at a concentration of 10 mM, both in tris-buffered saline (50 mM Tris, 150 mM NaCl, pH = 7.4). In the case of ANT and HBA, higher concentrations of ligand were also tested though again no binding was observed. Titration reactions were performed with 28 injections, all 10 µl in volume, with constant stirring at 300 rpm at 25^{o}C. Data acquisition and binding coefficients were determined with the Origin-based MicroCal analysis software.

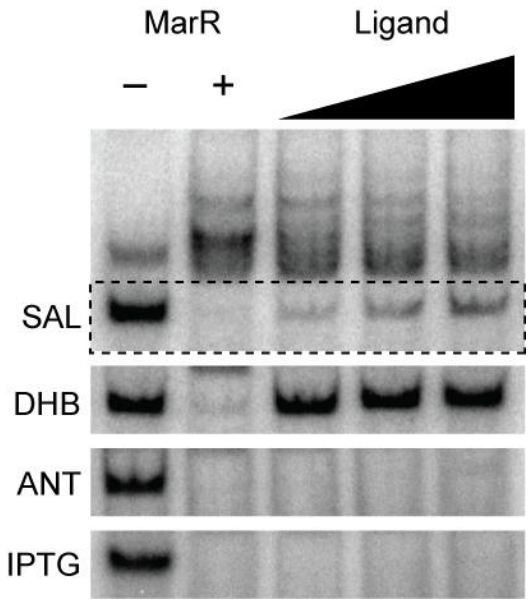

Figure 6.3. MarR DNA binding activity in the presence of aromatic acid metabolites as determined by electromobility shift assays. Binding reactions consisted of 20 ng of purified MarR and 5 ng of a radiolabeled, 150 bp fragment of the *marRAB* promoter containing two MarR operator sites. Ligands salicylate (SAL), 2,3-dihydroxybenzoate (DHB), anthranilate (ANT) and IPTG were supplied at increasing concentrations of 2.5 mM, 5 mM, and 10 mM to binding reactions. Reactions were displayed on 5% acrylamide, 0.5X TBE buffered gels. Loss of MarR DNA binding activity was monitored by the emergence of free DNA in the presence of these ligands. Salicylate and IPTG served as positive and negative controls respectively.

Chapter 7: Conclusions and Future Directions

7.1 General conclusions

In this work, an integrated, testable regulatory model for the *mar/sox/rob* network has been established. In the process of defining an integrated model we also confirmed that the global regulator CRP binds to the P_{marR} promoter directly. An important consequence of this finding is that it appears information regarding the nutritional state of the cell is fed into the *marRAB* circuit; in addition to numerous exogenous chemical signals that are known to influence expression of the *mar/sox/rob* regulon. We also were able to use the model to test predictions about the behavior of a feedforward regulatory loop formed by Rob and MarA. The outcome of this examination was the discovery that MarA acts as a conditional, negative autoregulator in the presence of Rob. We propose that this mechanism serves to establish set-point control on *marA* gene expression.

Using the interconnected model as a basis we were also able to explore the role of the *mar/sox/rob* regulatory circuits in altering outer membrane porin compositions. Specifically, we were able to demonstrate that it is through the combined action of Rob and *marRAB* that OmpF expression is reduced in the presence of salicylate. Additionally, we were able to show that the $OmpF^-$ phenotype observed in *tolC* mutants is the result of Rob activation alone. These

results highlight the overlapping nature of the *mar/sox/rob* regulon and that to produce similar output phenotypes under different conditions requires a fully intact regulatory network.

Finally, we were able to demonstrate that intermediary metabolites in the superpathway of chorismate are capable of activating the *marRAB* genetic system. In particular, the enterobactin intermediate, 2,3-dihydroxybenzoate, is capable of binding to MarR directly, affecting DNA binding activity. Further, we provided genetic evidence that disruptions in enterobactin biosynthesis can contribute, in part, to the upregulated P_{marR} activity observed in *tolC* mutants. Collectively, these results point to the necessity of examining intermediary metabolites as inducers of intrinsic multiple antibiotic resistance. Further, these results supply evidence for arguments that multidrug efflux systems may have evolved to combat the accumulation of metabolites and not necessarily antibiotics.

7.2 Future directions

Metabolite inducers of Rob. The results of Chapter 5 clearly show that Rob is the primary mediator of OmpF downregulation in *tolC* mutants. However, these results do not point to the true cause of Rob activation. As we have shown metabolites are capable of inducing the *marRAB* system in Chapter 6, it is easy to imagine that metabolites may also be activators of Rob. To test this

hypothesis, we propose that transposon mutagenesis and screening be done in a *tolC* genetic background where all three regulatory systems are removed (strain LC539) and harbors a fusion of *lacZ* to P_{micF}. Additionally, Rob is expressed ectopically by a constitutive promoter. Based on our observations, in mutants lacking *marRAB*, *soxRS*, and *rob* the P_{micF} promoter is severely downregulated in all medias explored. In these mutagenesis experiments, one would look for mutants with a downregulated P_{micF} phenotype (in this case $LacZ^-$). Ectopic expression of Rob will control for P_{rob} dependent expression effects as well as reduce the false positive rate produced by insertions made in the *rob* coding region. Considering our data, this screen appears entirely possible and will likely yield a number of possible new avenues for further exploration.

A high-throughput screen for MarR substrates. We have demonstrated in Chapter 6 that sufficient quantities of MarR can be purified under native conditions to perform biochemical analysis and binding assays such as isothermal titration calorimetry (ITC). Utilizing high-throughput methods such as the recently developed photonic crystal biosensor assay for DNA binding proteins would allow large chemical libraries to be screened against MarR to find additional activators. As the substrate specificity of MarR appears to be broad, yet somewhat specific (see Chapter 6), identifying larger families of chemical

structures that MarR will bind may allow for the “true” inducers of MarR to be systematically identified.

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Author's biography

Lon Michael Chubiz was born in Fridley, Minnesota on June 13th, 1981. His parents, Michael and Anne, moved to Blaine, Minnesota (a northern suburb of Minneapolis) shortly thereafter. There, Lon was raised there and attended Blaine High School, where he graduated in the spring of 2000. The following fall, Lon attended courses at the University of Minnesota ('The U'), where he later completed his Chemical Engineering Bachelors degree (B.Ch.E.) in the Department of Chemical Engineering and Material Science. During his time at the University of Minnesota, he worked as a research technician at 3M Company under the guidance of Dr. Prabhakara S. Rao, as well as undergraduate research with Dr. Yiannis Kaznessis at 'The U'. After strong encouragement from both these mentors, Lon decided to persue a doctoral degree in chemical engineering at the University of Illinois at Urbana-Champaign. There, he began his dissertation studies with Dr. Christopher V. Rao, examining regulatory mechanism in multidrug resistance and fundamental aspects of bacterial, ribosomal translation. Following the completion of his Ph.D. he is to begin post-doctoral training in the laboratory of Dr. Christopher J. Marx at Harvard University in the Department of Organismal and Evolutionary Biology examining evolutionary trajectories in metabolic adaptation. Lon's long-term plans are to seek a faculty position at a major research university.